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13. ABSTRACT (Maximum 200 Words)

The findings in this report have demonstrated that GADD45, a stress-inducible gene that plays an important role in cellular response to DNA damage, is regulated by tumor suppressor BRCA1 and involved in the control of cell cycle G2-M checkpoint. Through a series of molecular and biochemical approaches, we have found that BRCA1 transcriptionally regulates GADD45. BRCA1 activation of the GADD45 promoter was mediated through the OCT-1 and CAAT motifs located at the GADD45 promoter region. Site-directed mutations of both OCT-1 and CAAT motifs abrogats induction of the GADD45 promoter by BRCA1. BRCA1 has been found to associate with the Gadd45 promoter, through physical interaction with Oct-1 and NF-YA, which directly bind to the Gadd45 promoter. Importantly, we have demonstrated that inducible expression of Gadd45 protein results in cell cycle G2/M arrest. Gadd45-induced G2-M arrest requires normal cellular p53 function, but is independent of p38 kinase activity. Interestingly, induction of Gadd45 protein is coupled with reduction of nuclear cyclin B1 protein, whose nuclear localization is critical for the completion of G2-M transition. The reduced nuclear cyclin B1 levels are correlated with inhibition of Cdc2/cyclin B1 kinase activity. This study demonstrates a novel pathway (BRCA1-GADD45) involved in cellular response to DNA damage.

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1. INTRUDUCTION

Breast cancer is the most frequent malignancy in women. More than half of the hereditary human breast cancer can be attributed to mutations in the breast susceptibility gene BRCA1 (1-3). BRCA1 has been implicated in cellular response to DNA damage, including cell cycle checkpoints, apoptosis and DNA repair (4-10). These biological events are thought o maintain genomic stability. Deregulation of genomic fidelity is closely associated with malignant transformation and tumorigenesis. However, the molecular mechanism by which BRCA1 plays a role in maintaining genomic integrity remains to be elucidated. This is to define the molecular pathway, which mediates BRCA1's role in the control of cell cycle G2-M checkpoint. The proposed studies will provide the understanding of how BRCA1 participates in maintenance of genomic stability and prevents the onset of breast cancer, as well as provide the insight to development of novel new anticancer drugs. Our previous findings indicate that BRCA1 transcriptionally activates Gadd45, a p53-regulated and DNA damage-inducible gene that may play an important role in cell cycle G2-M checkpoints, apoptosis and DNA repair in response to DNA damage (11-17). Therefore, we speculated that the role of BRCA1 in cell cycle G2-M checkpoint might be mediated through Gadd45. Therefore, two major tasks have been proposed in this study. (1). To define the role Gadd45 in BRCA1-induced cell cycle G2-M arrest. (2). To determine the biochemical and molecular mechanism by which BRCA1 regulates Gadd45.

2. BODY

During the entire funding period of this grant, significant progress has been achieved. All proposed work has been well completed. These results are summarized as the following individually.

In Task 1:

1. To study the role of Gadd45 in the BRCA1-induced cell cycle G2-M checkpoint, we have successfully developed two GADD45-inducible cell lines via tet-off system in Hela (p53 negative status) and HCT116 (with wt p53) cells. In these cell lines, expression of Gadd45 protein is precisely controlled through the presence of tetracycline. Following withdraw of tetracycline, Gadd45 protein is highly induced (**results are shown in *Oncogene* paper in the appendix 1**).
2. Using these cell lines, we have found that induction of Gadd45 protein significantly suppresses cell growth (**results are shown in *Oncogene* paper in the appendix 1**).
3. We have found that induction of Gadd45 protein in HCT116 cell line (wt p53) results in cells arrest in G2-M phase, but induction of Gadd45 protein in Hela cells (deleted p53) failed to generate G2-M accumulation, suggesting that the Gadd45-activated cell cycle G2-M arrest is dependent of normal cellular p53 function (**results are shown in *Oncogene* paper in the appendix 1**).
4. Gadd45-induced cell cycle G2-M arrest is independent of p38 kinase activity, which is required for the initiation of G2-M checkpoint (**results are shown in *Oncogene* paper in the appendix 1**).

5. Inducible expression of Gadd45 protein is shown to alter the subcellular distribution of cyclin B1 and results in reduction of nuclear cyclin B1 levels. These alterations are coupled with inhibition of Cdc2/cyclin B1 kinase activity (**results are shown in *Oncogene* paper in the appendix 1**).
6. We have used siRNA approaches to suppress endogenous BRCA1 protein and carried out analysis on cell cycle progression. Our results demonstrate that the UV-induced cell cycle checkpoint in cells treated with BRCA1 siRNA was impaired, suggesting that BRCA1 is an important player in cell cycle G2-M checkpoint (**Manuscript in preparation**).
7. We have introduced the BRCA1 expression vector into Gadd45 deficient cells (ras-immortalized embryonic mouse fibroblast derived from gadd45 knockout mice) and examined the growth suppression. We have found that BRCA1-induced growth suppression is partially abrogated in Gadd45-deficient cells, indicating that Gadd45 plays a role in mediating growth inhibition by BRCA1. In addition, we have found BRCA1-induced cell cycle G2-M arrest is deficient in Gadd45 deficient cells (**Manuscript in preparation**).
8. Therefore, these results demonstrate that Gadd45, as a BRCA1-targeted effector, is capable of mediating BRCA1-induced cell cycle arrest.

In Task 2:

1. We have found expression of BRCA1 is able to upregulate Gadd45 mRNA when BRCA1 expression vector is introduced into human breast carcinoma MCF-7 cells (**results are shown in *Oncogene* paper in the appendix 2**).

2. We have demonstrated that BRCA1 can activate the Gadd45 promoter and BRCA1 activation of the Gadd45 promoter requires normal transcriptional activity of BRCA1 since the tumor-derived mutants of BRCA1, which lack normal transcriptional property, are unable to induce the Gadd45 promoter (**results are shown in *Oncogene* paper in the appendix 2**).

3. We have further demonstrated that BRCA1 activation of the Gadd45 promoter is a BRCA1-mediated specific effect (**results are shown in *Oncogene* paper in the appendix 2**).

4. Importantly, we have mapped the BRCA1-regulatory elements in the Gadd45 promoter. These BRCA1-responsive elements are localized at the region of the Gadd45 promoter between –107 to –57. Deletion of this region has been shown to abrogate BRCA1 activation of the Gadd45 promoter (**results are shown in *Oncogene* paper in the appendix 2**).

5. Based on the sequence analysis, we have found that there are two OCT-1 motifs and one CAAT box located at the region of the Gadd45 promoter from –107 to –57. Disruption of these motifs abolishes activation of the Gadd45 promoter by BRCA1 (**results are shown in JBC paper in appendix 4**).

6. Using a "pull-down" assay, we have shown that BRCA1 protein binds to the Gadd45 promoter. This binding is mediated through both Oct-1 and NF-YA (CAAT box binding protein) transcription factors because depletion of these proteins results in abrogation of BRCA1 protein binding to the Gadd45 promoter DNA (**results are shown in JBC paper in the appendix 4**).

7. In immunoprecipitation assays, the BRCA1 protein has been shown to physically interact with Oct-1 and NF-YA proteins (**results are shown in JBC paper in the appendix 4**).

8. Therefore, these results demonstrate that Gadd45 is one of the BRCA1-targeted genes in cellular response to DNA damge.

3. KEY RESEARCH ACCOMPLISHMENTS

The key accomplishments of this project over the past year include: (1). We have made first demonstration that BRCA1 activates the promoter of the Gadd45 gene that play an important role in cell cycle checkpoint, apoptosis and DNA repair. These results indicate that implication of BRCA1 in transcriptional regulation may contribute to its role as a tumor suppressor in maintaining genomic stability. (2). We have made first demonstration that BRCA1 play a role in gene regulation through OCT-1 and CAAT box. This finding will significantly broaden the role of BRCA1 in regulation of its targeted genes. (3). We have demonstrated that Gadd45 is a key player in the control of cell cycle G2-M arrest and is capable of mediating BRCA1's role in the control of cell cycle progression.

4. REPORTABLE OUTCOMES

The reportable outcomes related to this project include:

(1). Three papers have been published in ONCOGENE, which is a prestigious journal in the cancer research field (Appendix 1-3).

(2). One paper has been published in J. Bio Chem (Appendix 4).

(3). One poster presentation was taken place in the Annual meeting of the American Association for Cancer Research, at New Orleans, 2001 (Appendix 5).

(4). Abstract presented in 2001 annual AACR meeting (Appendix 6).

(5). Abstract presented in the meeting "Era of hope", 2002 (Appendix 7).

(6). We have established two Gadd45 tet-off inducible cell lines, which are useful resources and available to the scientists in this field.

(7). Under the support of this grant, one postdoctoral fellow is hired to obtain comprehensive training in my laboratory.

5. CONCLUSION

Overall, this DOD-funded project has been well performed during the entire funding period. The findings from this project have demonstrated a novel pathway (BRCA1-Gadd45) in cellular response to DNA damage, indicating that role of BRCA1 in maintenance of genomic stability may be mediated through Gadd45. Detailed information has been explored to define the biochemical and molecular mechanism by which the BRCA1-Gadd45 pathway plays important role in preventing breast cancer. The observations in this project may provide insight into development of new anti-breast cancer drugs.

6. REFERENCES

1. Easton DF. Ford D. Bishop DT. Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. American Journal of Human Genetics. *1*:265-71, 1995

2. Ford D. Easton DF. The genetics of breast and ovarian cancer. British Journal of Cancer. *4*:805-12, 1995 Oct

3. Miki Y. Swensen J. Shattuck-Eidens D. Futreal PA. Harshman K. Tavtigian S. Liu Q. Cochran C. Bennett LM. Ding W. et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science. *5182*:66-71, 1994

4. Gowen LC. Avrutskaya AV. Latour AM. Koller BH. Leadon SA. BRCA1 required for transcription-coupled repair of oxidative DNA damage. Science. *5379*:1009-12, 1998

5. Somasundaram K. Zhang H. Zeng YX. Houvras Y. Peng Y. Zhang H. Wu GS. Licht JD. Weber BL. El-Deiry WS. Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. Nature. *6647:*187-90, 1997

6. Scully R. Chen J. Ochs RL. Keegan K. Hoekstra M. Feunteun J. Livingston DM. Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *3:*425-35, 1997

7. Shao, N., Chai, Y. L., Shyam, E., Reddy, P., and Rao, V. N. Induction of apoptosis by the tumor suppressor protein BRCA1, Oncogene. *13:* 1-7, 1996.

8. Fan, S., Wang, J. A., Yuan, R. Q., Ma, Y. X., Meng, Q., Erdos, M. R., Brody, L. C., Goldberg, I. D., and Rosen, E. M. BRCA1 as a potential human prostate tumor suppressor: modulation of proliferation, damage responses and expression of cell regulatory proteins, Oncogene. *16:* 3069-82, 1998.

9. Thangaraju, M., Kaufmann, S. H., and Couch, F. J. BRCA1 facilitates stress-induced apoptosis in breast and ovarian cancer cell lines. J Biol Chem. *275:* 33487-96, 2000.

10. Shen SX. Weaver Z. Xu X. Li C. Weinstein M. Chen L. Guan XY. Ried T. Deng CX. A targeted disruption of the murine Brca1 gene causes gamma-irradiation hypersensitivity and genetic instability. Oncogene. *24:*3115-24, 1998

11. Kastan MB. Zhan Q. el-Deiry WS. Carrier F. Jacks T. Walsh WV. Plunkett BS. Vogelstein B. Fornace AJ Jr. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *4:*587-97, 1992

12. Zhan Q. Bae I. Kastan MB. Fornace AJ Jr. The p53-dependent gamma-ray response of GADD45. Cancer Res. *10:*2755-60, 1994a

13. Wang X-W. Zhan Q. Coursen JD. Khan M. Kontny U. Yu L. Hollander CM. O'Connor PM. Fornace AJ and Harris CC. GADD45 induction of a G2-M cell cycle checkpoint. Proc Natl Acad Sci USA. *96:*3706-3711, 1999

14. Smith ML. Chen IT. Zhan Q. Bae I. Chen CY. Gilmer TM. Kastan MB. O'Connor PM. Fornace AJ Jr. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science. *5189:*1376-80, 1994

15. Smith ML. Kontny HU. Zhan Q. Sreenath A. O'Connor PM. Fornace AJ Jr. Antisense GADD45 expression results in decreased DNA repair and sensitizes cells to u.v.-irradiation or cisplatin. Oncogene. *10:*2255-63, 1996

16. Takekawa M and Saito H. A family of stress-inducible Gadd45-like proteins mediate activation of the stress-responsive MTK/MEKK4 MAPKKK. *95:* 521-530, 1998

17. Hollander, M. C., Sheikh, M. S., Bulavin, D. V., Lundgren, K., Augeri-Henmueller, L., Shehee, R., Molinaro, T. A., Kim, K. E., Tolosa, E., Ashwell, J. D., Rosenberg, M. P., Zhan, Q., Fernandez-Salguero, P. M., Morgan, W. F., Deng, C. X., and Fornace, A. J., Jr. Genomic instability in Gadd45a-deficient mice, Nat Genet. *23:* 176-84, 1999.

Appendices

1. **Reprint published in Oncogene (Jin et al 2002)**
2. **Reprint published in Oncogene (Jin et al 2001)**
3. **Reprint published in Oncogene (Jin et al 2003)**
4. **Reprint published in JBC (Fan et al, 2002)**
5. **Abstract presented in the 93rd annual AACR meeting, 2001**
6. **Abstract presented in the 94th annual AACR meeting, 2002**
7. **Abstract presented in the meeting "Era of hope", 2002.**

Appendix 1

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GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity

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In response to DNA damage, the cell cycle checkpoint is an important biological event in maintaining genomic fidelity. Gadd45, a p53-regulated and DNA damage inducible protein, has recently been demonstrated to play a role in the G2-M checkpoint in response to DNA damage. In the current study, we further investigated the biochemical mechanism(s) involved in the *GADD45*-activated cell cycle G2-M arrest. Using the tetracycline-controlled system (tet-off), we established *GADD45*-inducible lines in HCT116 (wild-type p53) and Hela (inactivated p53 status) cells. Following inducible expression of the Gadd45 protein, cell growth was strongly suppressed in both HCT116 and Hela cells. Interestingly, HCT116 cells revealed a significant G2-M arrest but Hela cells failed to arrest at the G2-M phases, indicating that the *GADD45*-activated G2-M arrest requires normal p53 function. The *GADD45*-induced G2-M arrest was observed independent of p38 kinase activity. Importantly, induction of Gadd45 protein resulted in a reduction of nuclear cyclin B1 protein, whose nuclear localization is critical for the completion of G2-M transition. The reduced nuclear cyclin B1 levels correlated with inhibition of Cdc2/cyclin B1 kinase activity. Additionally, overexpression of cyclin B1 substantially abrogated the *GADD45*-induced cell growth suppression. Therefore, *GADD45* inhibition of Cdc2 kinase activity through alteration of cyclin B1 subcellular localization may be an essential step in the *GADD45*-induced cell cycle G2-M arrest and growth suppression. *Oncogene* (2002) **21,** 8696–8704. doi:10.1038/sj.onc.1206034

Keywords: p53; *GADD45*; G2-M arrest; cyclin B1

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Introduction

In response to DNA damage, mammalian cells arrest at the transition from G1 to S phase (G1-S checkpoint) and G2 to M phase (G2-M checkpoint) (Hartwell and Weinert, 1989). Cell cycle arrest at these checkpoints prevents DNA replication and mitosis in the presence of DNA damage. Inactivation of those cell cycle checkpoints results in genomic instability, which is closely associated with cell transformation and tumorigenesis. In addition, disruption of normal cell cycle controlling machinery often has dramatic consequences on therapeutic sensitivity (Elledge, 1996; Hartwell and Kastan, 1994; Kohn *et al.*, 1994; O'Connor and Kohn, 1992; Paulovich *et al.*, 1997).

Currently, the mechanism(s) by which DNA damaging agents activate cell cycle G1-S checkpoint is well understood. The tumor suppressor p53 gene plays a critical role in the control of G1-S arrest. Following DNA damage, p53 transcriptionally up-regulates p21 (el-Deiry *et al.*, 1993), one of the p53-downstream genes and a potent cell cycle-dependent kinase inhibitor. Subsequently, induced p21 forms complexes with Cdk-cyclin and inhibits the activity of cdk4-cyclin D, Cdk6-cyclin D, Cdk2-cyclin E, and Cdk2-cyclin A, and in turn transiently arrest cells at the G1-S transition (Harper *et al.*, 1993; Sherr and Roberts, 1995; Xiong *et al.*, 1993; Zhang *et al.*, 1994). It has been demonstrated that the disruption of endogenous p21 abrogates the G1-S checkpoint after cell exposure to DNA damage (Waldman *et al.*, 1995). P53 has also been implicated in the control of the G2-M checkpoint. Introduction of p53 into p53-deficient human fibroblasts results in both G1-S and G2-M arrest (Agarwal *et al.*, 1995; Stewart *et al.*, 1995) and the HPV-16 E6 viral oncoprotein, which blocks p53 function, has been shown to decrease the stringency of the mitotic checkpoint (Thompson *et al.*, 1997). Recent evidence indicates that p53 and p21 are required for maintaining the G2 checkpoint in human HCT116 cells (Bunz *et al.*, 1998). In addition, 14-3-3, which blocks Cdc25 activity and arrests cells at the G2-M transition, is demonstrated as one of the p53 downstream genes

(Hermeking *et al.*, 1997). Most recently, *GADD45*, a p53-regulated and DNA damage-inducible gene, is found to play an important role in the G2-M checkpoint in response to certain types of DNA damaging agents (Jin *et al.*, 2000; Wang *et al.*, 1999; Zhan *et al.*, 1999).

However, the G2-M checkpoint is complex and may involve redundant controls including both p53-independent and p53-dependent mechanisms. It has been well accepted that many of the G2-M regulators appear to ultimately target Cdc2, a protein kinase required for the mitotic entry in mammalian cells (Elledge, 1996; O'Connor, 1997). Activation of Cdc2 kinase requires its association with mitotic cyclins (cyclin B1 and cyclin A), and phosphorylation at Thr-161 and dephosphorylation at Thr-14/Tyr-15 cells (Ducommun *et al.*, 1991; Elledge, 1996; O'Connor, 1997). After DNA damage, several G2-M regulators, including Chk1, Chk2, 14-3-3 and ATM, alter Cdc2 activity by inhibiting dephosphorylation of Cdc25C phosphatase. The inhibition of Cdc25C activity prevents the removal of inhibitory phosphorylations from Thr-14 and Tyr-15 of Cdc2 (Elledge, 1996; O'Connor and Fan, 1996; Paulovich *et al.*, 1997). In addition, DNA damage is able to suppress Cdc2 activity by inhibiting the accumulation of cyclin B1 mRNA and protein (Bernhard *et al.*, 1995; Muschel *et al.*, 1991, 1992). Delayed entry into mitosis following DNA damage also correlates with nuclear exclusion of cyclin B1 protein (Toyoshima *et al.*, 1998).

The *GADD45* gene is induced by a variety of DNA damaging agents, including ionizing radiation (IR), methyl methansulfonate (MMS), UV radiation (UV), hydroxyurea and medium starvation (Fornace *et al.*, 1988, 1989; Papathanasiou *et al.*, 1991). The IR-induction of *GADD45* is transcriptionally regulated by p53 via a p53-binding site in the third intron (Kastan *et al.*, 1992; Zhan *et al.*, 1994a). In contrast, *GADD45* induction by UV radiation or MMS treatment is detected in all mammalian cells regardless of p53 status. However, recent evidence shows that p53 can still contribute to cellular responses to UV, MMS and medium starvation although it is not required (Zhan *et al.*, 1996, 1998). Gadd45 is a nuclear protein and binds to multiple important cellular proteins such as proliferating cell nuclear antigen (PCNA) (Hall *et al.*, 1995; Smith *et al.*, 1994), p21 protein (Chen *et al.*, 1996; Kearsey *et al.*, 1995; Zhao *et al.*, 2000), core histone protein (Carrier *et al.*, 1999), MTK/MEKK4 (Takekawa and Saito, 1998), an upstream activator of the JNK pathway, and Cdc2 protein kinase. The presence of Gadd45 in these complexes indicates that Gadd45 may be an important player in cell cycle control, DNA repair and the regulation of signaling pathway. The role of *GADD45* in maintaining genomic stability has been demonstrated by the recent finding that the mouse embryonic fibroblasts (MEF), derived from gadd45-null mice exhibit aneuploidy, chromosomal aberrations, gene amplification and centrosome amplification. Additionally, gadd45-knockout mice display increased radiation carcinogenesis (Hollander *et al.*, 1999). In this study, we have further investigated the role of *GADD45* in the G2-M checkpoint and demonstrated that the *GADD45*-induced G2-M arrest depends on normal cellular p53 function, but is independent of p38 kinase activity, which is reported to be required for the initiation of the G2-M checkpoint after UV radiation. In addition, inducible expression of Gadd45 protein has been shown to result in alterations of cyclin B1 subcellular distribution, which might be a consequence of the interaction of Gadd45 with Cdc2 proteins.

Results

Inducible expression of Gadd45 protein suppresses human cell growth

To further investigate the biological mechanism(s) by which *GADD45* plays a role in the control of cell cycle regulation, we established tetracycline-regulated *GADD45*-inducible cell lines in human cervical cancer Hela cells (see Materials and methods), where cellular p53 function is inactivated, and human colorectal carcinoma HCT116 cells, which has wild-type p53 and normal p53 function. As shown in Figure 1a, both Hela *GADD45*-inducible cells and HCT116 *GADD45*-inducible cells exhibited extremely low basal levels of the endogenous Gadd45 protein. Following withdrawal of tetracycline, Gadd45 protein was greatly induced and presented more than 10-fold induction in both cell lines. Next, the effect of Gadd45 protein on growth suppression was examined in these two *GADD45*-inducible cell lines. To perform this experiment, 500, 1000 or 2000 cells were seeded and grown in DMEM medium at 100-cm dishes 16 h prior to tetracycline withdrawal. After removing tetracycline, cells continued to grow for 14 days and then were fixed, scored for colonies containing more than 50 cells. Similar to our previous finding that overexpression of *GADD45* protein via transient transfection inhibits tumor cell growth (Zhan *et al.*, 1994b), inducible expression of Gadd45 protein in both Hela and HCT116 lines strongly suppressed colony formation (Figure. 1B), indicating a suppressive role of *GADD45* in cell growth regardless of p53 status. In agreement with this observation, both Hela and HCT116 lines with inducible expression of Gadd45 protein exhibited a substantial slow growth rate (results not shown). Taken together, these results indicate that *GADD45* plays a negative role in the control of cell progression.

GADD45 induced cell cycle G2-M arrest depends on normal cellular p53 function

In order to further determine the role of *GADD45* in the control of cell cycle G2-M arrest, cell cycle distribution analyses were conducted in both HCT116 and Hela *GADD45*-inducible cell lines. Following removal of tetracycline, *GADD45*-inducible cells were collected at 24 h or 36 h and subject to flow cytometry

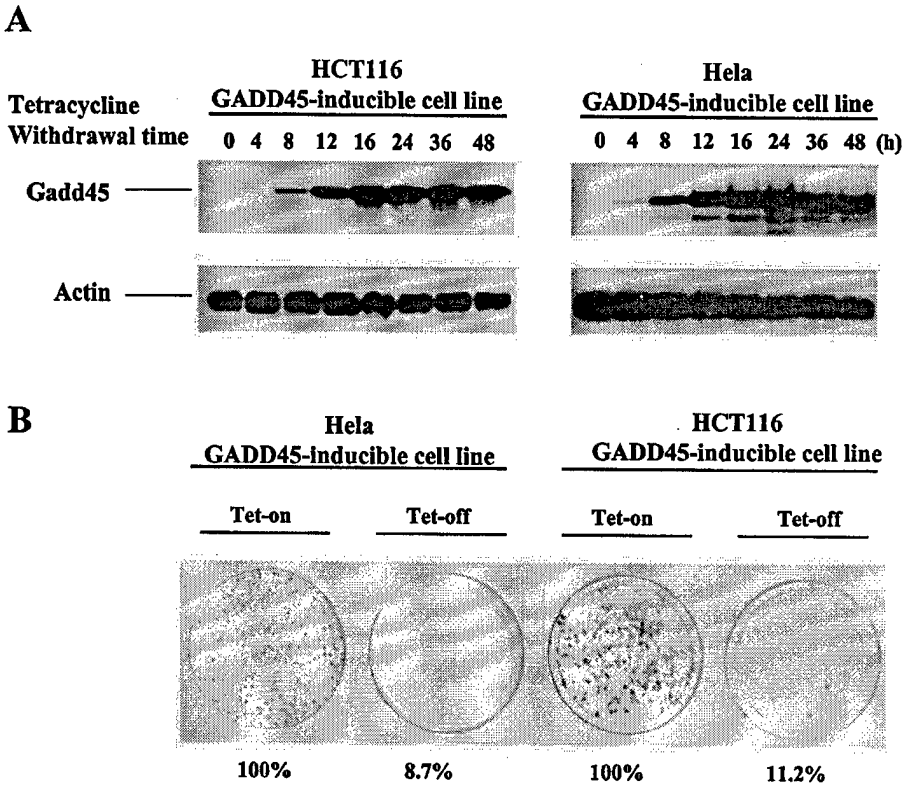

Figure 1 Tumor cell growth suppression by Gadd45 protein. (a) Induction of Gadd45 protein in HCT116 and Hela cells controlled by the Tet-off system. HCT116 *GADD45*-inducible lines and Hela *GADD45*-inducible cell lines were established as described in Materials and methods. Cells were placed in 100 mm dishes at a density of 4×10^5 and grown in DMEM medium containing tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were collected at the indicated time points for preparation of cellular protein. 100 μg of whole cell protein was used for immunoblotting analysis with anti-*GADD45* antibody. As a loading control, anti actin antibody was included. (b) Induction of Gadd45 protein suppresses cell growth. HCT116 and Hela *GADD45*-inducible cells were seeded at a density of 1000 cells per 100 mm dish and grown in medium containing 2 μg/ml of tetracycline. After 16 h, medium was removed and plates were washed three times with PBS, then fresh medium containing no tetracycline was added into plates. The cells were fixed and stained at 14 days and scored for colonies containing at least 50 cells. The experiments were performed four times and only representative results were shown here

analysis. The results presented in Figure 2 depict a representative profile of cell cycle distribution in those cells. In the HCT116 *GADD45*-inducible line (wt p53 status), cells grown in the presence of tetracycline presented 14–16% population in G2-M phase. However, inducible expression of Gadd45 protein resulted in a clear accumulation of the G2-M fraction. Approximately 32% of the cells were arrested at the G2-M phase of the cell cycle in the absence of tetracycline, indicating that *GADD45* expression alone is able to halt cells in G2-M phase. In contrast, after inducible expression of Gadd45, Hela cells (inactivated p53 status) did not exhibit any evident changes of cell cycle distribution. In consistence with these results, introduction of *GADD45* expression vector into HCT116 via transient transfection resulted in increased G2-M population in HCT116 but not in HCT116 p53−/−, where p53 alleles were knocked out by homologous recombination approach (result not shown). These observations further demonstrate that *GADD45*-mediated G2-M arrest requires normal cellular p53 function.

GADD45 induced cell cycle G2-M arrest is not affected by inhibitors of p38

The mitogen-activated protein kinase p38 has recently been reported to play a critical role in cell cycle G2-M checkpoint in response to UV radiation (Bulavin *et al.*, 2001). To understand whether p38 kinase activation contributes to the *GADD45*-induced G2-M arrest, the mitotic index was measured in *GADD45* inducible cells in the presence of p38 kinase inhibitor, SB202190. In Figure 3a, high mitotic indices were observed in HCT116 cells treated with nocodazole. In response to UV radiation, mitotic indices substantially decreased, indicating that UV treatment arrests cells in the G-M transition. Addition of p38 inhibitor SB202190 at a concentration of 10 μM was shown to greatly attenuate the UV-induced G2-M arrest. In Figure 3b, inducible expression of Gadd45 protein exhibited low mitotic indices, which reflects a significant G2-M arrest by Gadd45. However, p38 inhibitor SB202190 (10 μM) showed little effect on the Gadd45-induced G2-M arrest. These results suggest that the *GADD45*

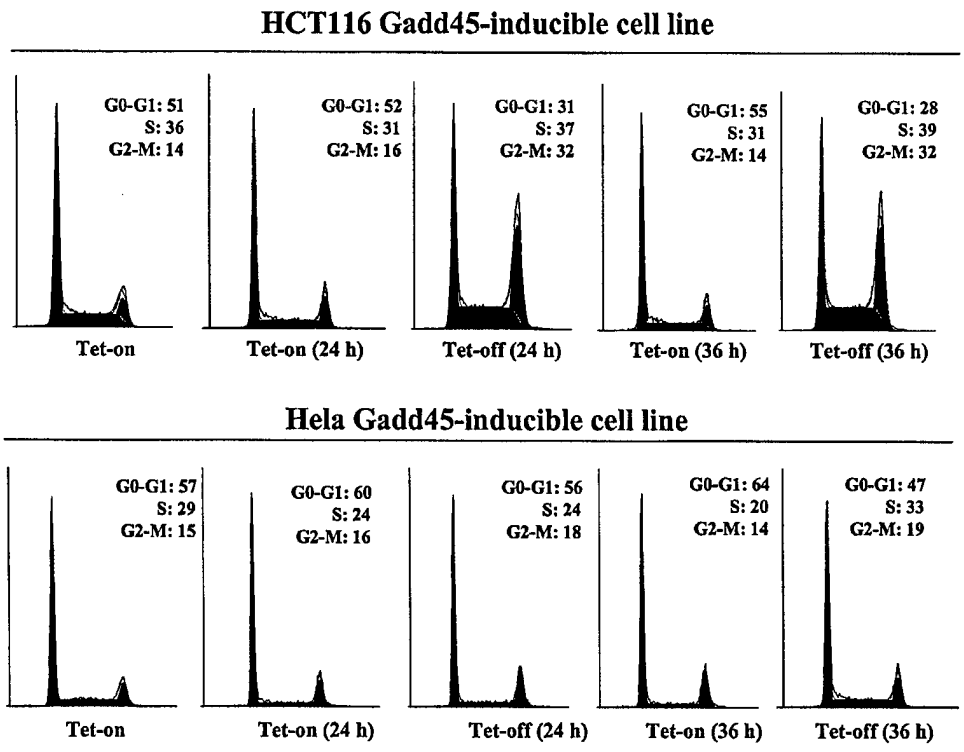

Figure 2 Cell cycle G2-M arrest following inducible expression of Gadd45 protein in both HCT116 and Hela cells. HCT116 and Hela *GADD45*-inducible cells were grown in DMEM medium with 10% fetal bovine serum in the presence of tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were collected at the indicated time points and subject to flow cytometric analysis as described in Materials and methods

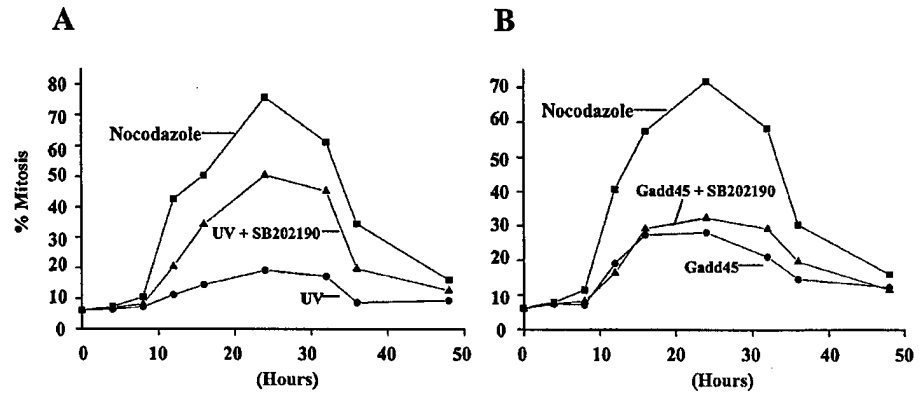

Figure 3 Mitotic entry after UV radiation or inducible expression of Gadd45 protein. (a) HCT116 cells were UV irradiated in the presence of 10 μM p38 kinase inhibitor SB203580 and mitotic indices were determined as described in Materials and methods. (b) HCT116 *GADD45*-inducible cells were grown in medium with tetracycline (2 μg/ml). Upon the withdrawal of tetracycline, cells were exposed to 10 μM p38 kinase inhibitor SB203580 and followed by determination of mitotic indices at the indicated time points

induction of cell cycle G2-M checkpoint does not require activation of p38 kinase.

Expression of Gadd45 protein alters the level of nuclear cyclin B1 but does not affect phosphorylation statuses of Cdc25C or Chk1

In our previous report, we have demonstrated that Gadd45 protein physically interacts with Cdc2 kinase, dissociates Cdc2/cyclin B1 complexes and in turn inhibits Cdc2 kinase activity, but does not alter Cdc2 phosphorylation status (Zhan *et al.*, 1999). However, the biochemical consequence of the interaction between Gadd45 and Cdc2 remains to be further defined. Since nuclear localization of cyclin B1 protein is thought to be critical for the completion of G2-M transition, we further examined cyclin B1 protein distributions in both the nucleus and cytoplasm. As shown in Figure

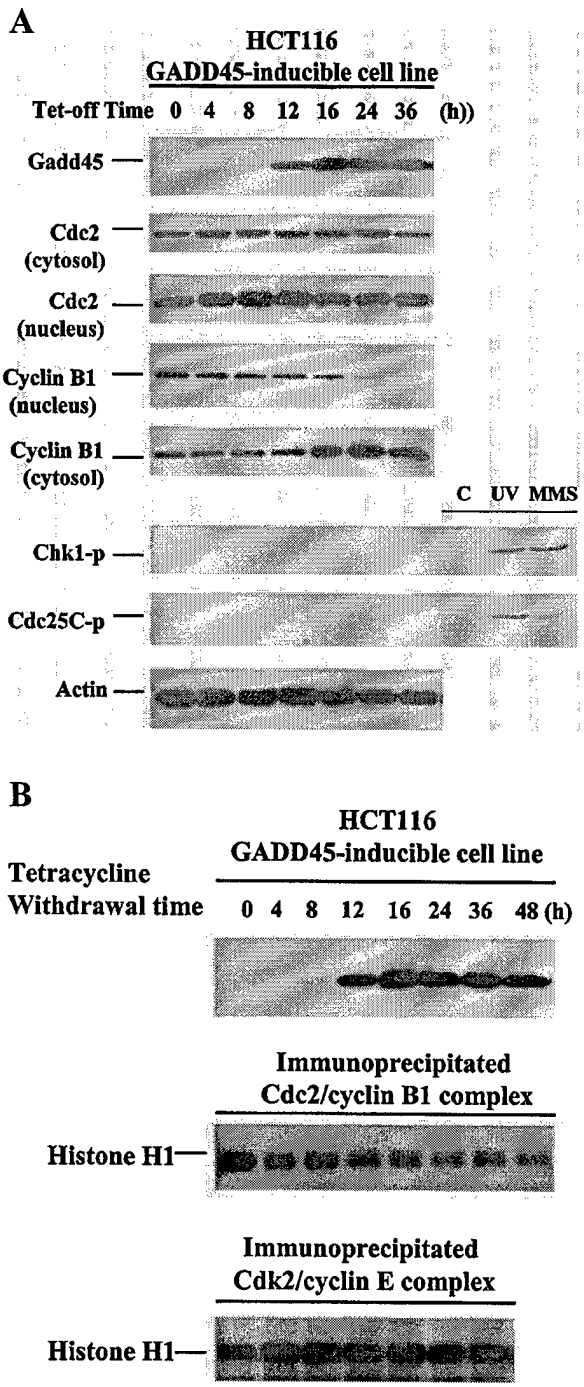

Figure 4 Subcellular localization of cyclin B1 protein and inhibition of Cdc2 kinase activity following inducible expression of Gadd45. (a) Cellular proteins were prepared from HCT116 *GADD45*-inducible cells after withdrawal of tetracycline at the indicated time points. 100 μg of proteins were loaded onto SDS–PAGE gel for detection of subcellular distribution of cyclin B1 protein and phosphorylations of CHK1 or Cdc25C. (b) One mg of cellular proteins isolated from HCT116 *GADD45*-inducible cells at the indicated time points was immunoprecipitated with anti-cyclin B1 or cyclin E antibodies, and histone H1 kinase assays were performed as described in Materials and methods. Labeled histone H1 was detected by autoradiography following size separation on a SDS–PAGE gel

4a, following inducible expression of Gadd45 protein in HCT116 cells, there were no evident alterations of Cdc2 protein in both the cytosol and nuclear compartments. Interestingly, nuclear cyclin B1 protein exhibited a significant reduction in response to induction of Gadd45. In support of this observation, cytosol cyclin B1 appeared to increase after Gadd45 induction. These results indicate that Gadd45 induction caused subcellular redistribution of cyclin B1 protein. In the same experiment, Chk1 and Cdc25C phosphorylation statuses were also examined, but no phosphorylations of Chk2 or Cdc25C were detected following Gadd45 induction. However, cells treated with UV and MMS displayed increased phosphorylations for Chk2 and Cdc25C. Additionally, we analysed Cdc2 and Cdk2 kinase activity following Gadd45 protein expression and found Cdc2 was inhibited by Gadd45 but Cdk2 kinase activity remained at the similar levels after Gadd45 expression (Figure 4b). Taken together, Gadd45 protein is able to alter cyclin B1 nuclear localization and in turn inhibits Cdc2 kinase activity.

Cyclin B1 abrogates the GADD45-induced cell growth suppression

We have previously demonstrated that *GADD45*-induced growth suppression (Zhan *et al.*, 1994b), in a great content, correlates with its inhibition of Cdc2/cyclin B1 kinase activity. It is assumed that interaction of Gadd45 with Cdc2 causes dissociation of the Cdc2/cyclin B1 complex, and in turn alters subcellular localization of cyclin B1, which contributes to the loss of Cdc2 kinase activity. Therefore, we examined whether introduction of cyclin B1 into cells can rescue *GADD45*-induced growth-suppression. To do this, a *GADD45* expression vector was co-transfected with expression vectors for cyclin B1, Cdc2, or cyclin D1 into HCT116 cells (p53 wt line). In Figure 5, expression of *GADD45* in HCT116 cells

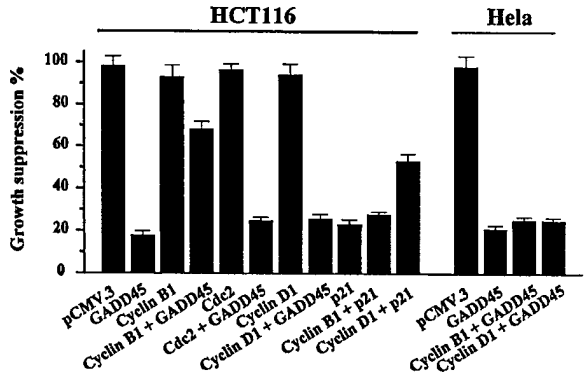

Figure 5 Effect of cyclin B1 expression on the *GADD45*-induced cell growth suppression. Human colorectal carcinoma HCT116 cells were transfected with the indicated expression vectors. Following selection with G418 for 2 weeks, cells were fixed and the colonies that contained at least 50 cells were counted. Quantitative results represent the average of three individual experiments

suppressed 80% cell growth. While in the presence of cyclin B1 overexpression, *GADD45* only generated 30% growth suppression, indicating that cyclin B1 is able to abrogate *GADD45*-induced growth inhibition. In contrast, overexpression of both Cdc2 and cyclin D1 failed to rescue the *GADD45*-induced growth suppression. Additionally, cyclin B1 had little effect on p21, suggesting that p21-induced cell growth might not be mainly through its role in the G2-M arrest. Interestingly, *GADD45* was able to suppress cell growth in Hela cells, which contains inactivated p53 and does not exhibit *GADD45*-induced G2-M arrest. However, both cyclin B1 and cyclin D1 were incapable of abrogating *GADD45*-induced cell growth, indicating *GADD45* inhibits cell growth in cells with abnormal p53 probably through a different mechanism distinct from the inhibition of Cdc2 kinase activity in p53 wt cell lines.

Discussion

In this report, we further investigated the role of Gadd45, a p53-regulated and stress-inducible protein, in the control of cell cycle G2-M checkpoint. Using tetracycline-controlling system (tet-off), we established *GADD45*-inducible lines in both HCT116 (wt p53) and Hela (negative p53 status) cells. Therefore, induction of Gadd45 protein was nicely manipulated by the withdrawal of tetracycline. Following inducible expression of Gadd45, cell growth was strongly inhibited in both HCT116 and Hela lines. In consistence with our previous finding that the introduction of *GADD45* expression vector into human normal fibroblast via microinjection approach causes cells to arrest at the G2-M transition, induction of Gadd45 protein in the HCT116 *GADD45*-inducible line greatly increased cell population in G2-M phase, but Gadd45 expression was unable to induce G2-M arrest in Hela cells, which contain inactivated p53. The *GADD45*-induced G2-M arrest appeared independent of p38 kinase activity, as employment of p38 kinase inhibitor (SB202190) did not abrogate GADD-induced G2-M arrest. More importantly, overexpression of Gadd45 protein was shown to result in reduction of nuclear cyclin B1 protein and inhibited Cdc2 kinase activity, but had no effect on Chk1, Cdc25C phosphorylation and Cdk2 activity. In addition, co-introduction of cyclin B1 expression vector was able to substantially disrupt the *GADD45*-induced growth suppression.

The tumor suppressor p53 gene has been implicated in the control of cell cycle checkpoint in response to genotoxic stress (Bunz *et al.*, 1998, 1999; Kastan *et al.*, 1991, 1992). The role for p53 in G1-S arrest is clearly shown to be mediated though p21 (Harper *et al.*, 1993; Sherr and Roberts, 1995; Xiong *et al.*, 1993; Zhang *et al.*, 1994). However, the role of p53 in the control of the G2-M arrest is under debate and remains to be further elucidated. It is postulated that as one of the p53-targeted genes (Kastan *et al.*, 1992; Zhan *et al.*, 1994a), *GADD45* might be a strong player in mediating p53-regulated cell cycle G2-M checkpoint. Previous studies have shown that Gadd45 protein interacts with Cdc2 and dissociates the Cdc2/cyclin B1 complex (Jin *et al.*, 2000; Zhan *et al.*, 1999). Subsequently, 'free' cyclin B1 protein dissociated from the Cdc2 complex is more likely pumped out from the nucleus, probably by the nuclear transport system. As a result of exclusion of cyclin B1 protein from the nucleus, Cdc2 kinase activity is inhibited and followed up by the cell cycle G2-M arrest. This goes along with the finding by Toyoshima *et al.* (1998) that DNA damage causes increased nuclear export of cyclin B1 and in turn arrests cells at the G2-M transition. Our observations that inducible expression of *GADD45* protein alters cyclin B1 nuclear localization (Figure 4) have suggested that exclusion of nuclear cyclin B1 protein by Gadd45 might be an essential step for the *GADD45*-induced G2-M arrest. Therefore, the findings in this work have further presented the precise evidence that the p53-*GADD45* pathway is well involved in the control of G2-M arrest.

The mechanism(s) for p53 dependence of the *GADD45*-induced cell cycle G2-M arrest is not clear at the present time. Bunz *et al.* (1998) has reported that cells with disrupted p53 display an impaired G2-M checkpoint after DNA damage, and suggested that the role for p53 in sustaining G2-M arrest after DNA damage might be mediated through p21. However, our previous investigations have already demonstrated that p21 is not required for *GADD45*-induced G2-M arrest, since introduction of *GADD45* expression vector into p21 deficient cells, where endogenous p21 has been disrupted, is able to generate G2-M arrest (Wang *et al.*, 1999). We have also not found any alterations of MDM2 protein level following Gadd45 induction and no physical interactions between Gadd45 and MDM2 proteins (result not shown). Therefore, both p21 and MDM2 appear not to be the candidates to mediate the role for p53 in *GADD45*-induced G2-M arrest. Future investigation is required to explore the mechanism by which p53 is required for the *GADD45*-induced G2-M arrest.

The mitogen-activated kinase p38 is required for initiating the G2-M checkpoint after UV radiation, probably through phosphorylating Cdc25B at serines 309 and 361 (Bulavin *et al.*, 2001). However, the *GADD45*-induced G2-M arrest is independent of p38 kinase activity. These results have further confirmed that Gadd45 acts at the late G2-M transition or early mitotic phase, instead of at the initiation of G2-M transition. In addition, the inhibitory effect of the Gadd45 protein appears to be specifically localized on Cdc2/cyclin B1 complex, as induction of Gadd45 protein does not alter phosphorylations of Chk1 and Cdc25C. Overexpression of cyclin B1 protein has been found in certain types of human tumors although the biological function of this overexpressed protein in tumorigenesis remains unclear (Soria *et al.*, 2000). Interestingly, Overexpression of cyclin B1 is closely associated with loss of a p53 function (Yu *et al.*, 2002). In Figure 5, co-expression of cyclin B1 with Gadd45

protein abrogated the Gadd45-induced cell growth suppression. This evidence has provided a new insight into understanding on the role of cyclin B1 in development of genomic instability and tumorigenesis.

GADD45 was shown to suppress cell growth in both HCT116 (wt p53) and Hela (inactivated p53) cells, regardless of p53 status (Figure 5). However, *GADD45* only generated G2-M arrest in HCT116 cells, but not in Hela cells, suggesting that the *GADD45*-induced growth suppression is complex and might involve the biological events distinct from the G2-M arrest. In fact, Takekawa and Saito (1998) have previously reported that *GADD45* interacts with MTK1/MEKK4, an upstream activator of the JNK pathways, and induced apoptosis in Hela cells. Therefore, *GADD45* is able to play a negative role in cell growth probably through both cell cycle arrest and apoptosis. The importance of *GADD45* in maintenance of genomic fidelity has been presented by the evidence that gadd45-null mice generated by gene targeting exhibit aneuploidy, chromosome aberrations, gene amplification and centrosome amplification, and increased tumorigenesis after DNA damaging agents (Hollander *et al.*, 1999). Therefore, the current studies have further demonstrated the mechanism(s) by which *GADD45* plays a role in maintaining genomic stability and provides insight into understanding the p53-*GADD45* pathway in cellular response to genotoxic stress.

Materials and methods

Establishment of the GADD45 inducible cell line and cell culture

To establish *GADD45* tet-off inducible cell lines, human colorectal carcinoma HCT116 cells were initially transfected with pTet-Off plasmid (Clontech, Palo Alto, CA, USA), which is commercially available and expresses the tTA regulator proteins, and the G418-resistant colonies were selected and amplified. Next, the cells expressing tTA proteins were subject to second round transfection with pTRE–*GADD45* construct, where the *GADD45* gene was inserted into *Bam*H1/*Hind*III sites of pTRE plasmid (Clontech). The cells transfected with pTRE-*GADD45* plasmid were selected by hygromycin at 200 μg/ml for 14 days, and each hygro-resistant colony was separately collected for detection of Gadd45 protein expression under tet-off system. In the case of Hela *GADD45* inducible lines, Hela cells expressing tTA were commercially obtained from Clontech and transfected with pTRE-*GADD45* construct and the hygro-resistant cells were selected as described in HCT116 cells. *GADD45*-inducible cells were grown in DMEM medium supplemented with 10% fetal bovine in the presence of tetracycline at a concentration of 2 μg/ml. To induce expression of Gadd45 protein, DMEM containing tetracycline was removed and the plates were washed four times with PBS, and fresh DMEM medium containing no tetracycline was then added to cells. Cells were collected at the indicated time points for examination of induced Gadd45 protein.

Antibodies and immunoblotting analysis

The following antibodies were used in the experiments; *GADD45*, Cdc2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin B1 (Pharmingen, San Diego, CA, USA) and Cdc25C and Chk1 phosphorylation-sites specific antibodies (Cell Signaling Technology Inc, Beverly, MA, USA). *GADD45*-inducible cells were exponentially grown in DMEM medium containing tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were collected at the indicated time. For preparation of cellular protein, plates were rinsed with PBS and cells were lysed in PBS containing100 μg/ml phenyl-methylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 1% NP-40 (lysis buffer). Lysates were collected by scraping and cleared by centrifugation at 4°C. 100 μg of cellular protein was loaded onto 12% SDS–PAGE gel and transferred to Protran membranes. Membranes were blocked for 1 h at room temperature in 5% milk, washed with PBST (PBS with 0.1% Tween-20), and incubated with indicated antibodies for 2 h. Membranes were washed four times in PBST and HRP-conjugated anti-mouse antibody was added at 1:4000 in 5% milk. After 1 h, membranes were washed and detected by ECL (Amersham, Arlington Height, IL, USA) and exposed to X-ray film (Kodak, Rochester, NY, USA).

Growth suppression assay

Five hundred, 1000, or 2000 cells from Hela or HCT116 *GADD45*-inducible lines were seeded in 100-cm dishes and grown in DMEM medium containing 2 μg/ml tetracycline for 16 h. Following withdrawal of tetracycline, cells were fixed at 14 days and scored for colonies containing at least 50 cells (Zhan *et al.*, 1994b).

Cdc2 and Cdk2 kinase assays

Cellular lysates isolated from the *GADD45*-inducible cells were incubated with 10 μl of cyclin B1 antibody (Pharmingen) or 20 μl of Cdk2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 20 μl of protein A/G agarose beads (Santa Cruz Biotechnology) at 4°C for 6 h. Immunocomplexes were washed four times with lysis buffer and followed by kinase buffer. Histone H1 kinase assays were then performed in the presence of 10 μg of histone H1 (Upstate Biotechnology, Lake Placid, NY, USA), 15 mM $MgCl_2$, 7 mM β-glycerol phosphate, 1.5 mM EDTA, 0.25 mM sodium orthvanadate, 0.25 mM DTT and 10 μCi of γ-^{32}P ATP in 30 μl volume. After 15 min at 30°C, the reactions were mixed with an equal amount of standard 2 × SDS protein denature loading buffer, sized-separated on a 12% SDS–PAGE gel (Zhan *et al.*, 1999).

Flow cytometry analysis

HCT116 and Hela *GADD45*-inducible cells were plated into 100-mm dishes at a density of 6×10^5 and grown in DMEM containing 2 μg/ml of tetracycline. Sixteen hours later, medium was removed and plates were washed four times followed by addition of fresh medium. After incubation for 36 h, cells were collected, washed with PBS, fixed with 70% ethanol for 2 h at 4°C. Cells were then incubated with RNase (10 μg/ml) for 30 min and stained with propidium iodine (Sigma; 50 μg/ml). Cell cycle analysis was performed using Becton Dickson fluorescence-activated cell analyzer. At least 10,000 FITC positive cells were analysed using CellQuest and Modfit programs (Wang *et al.*, 1999).

Analysis of mitotic index in HCT116 GADD45-inducible cells

HCT116 *GADD45*-inducible cells were seeded at a density of 6×10^5 in DMEM containing 2 μg/ml of tetracycline.

Following withdrawal of tetracycline, cells were grown in the presence of 10 μM p38 kinase inhibitor SB203580 and harvested at the indicated time points, fixed in methanol: acetic acid (3:1), spread on glass microscope slides, air-dried and stained with 5% Giemsa. Nuclei exhibiting condensed, evenly staining chromosomes were scored as mitotic. At least 1000 cells were counted in each determination. Meanwhile, HCT116 cells treated with p38 kinase inhibitor SB203580 were exposed to UV radiation and subjected to analysis of mitotic index.

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References

Agarwal ML, Agarwal A, Taylor WR and Stark GR. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 8493–8497.
Bernhard EJ, Maity A, Muschel RJ and McKenna WG. (1995). *Radiat. Environ. Biophys.*, **34**, 79–83.
Bulavin DV, Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, Appella E and Fornace Jr AJ. (2001). *Nature*, **411**, 102–107.
Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW and Vogelstein B. (1998). *Science*, **282**, 1497–1501.
Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, Lengauer C, Kinzler KW and Vogelstein B. (1999). *J. Clin. Invest.*, **104**, 263–269.
Carrier F, Georgel PT, Pourquier P, Blake M, Kontny HU, Antinore MJ, Gariboldi M, Myers TG, Weinstein JN, Pommier Y and Fornace Jr AJ. (1999). *Mol. Cell. Biol.*, **19**, 1673–1685.
Chen IT, Akamatsu M, Smith ML, Lung FD, Duba D, Roller PP, Fornace Jr AJ and O'Connor PM. (1996). *Oncogene*, **12**, 595–607.
Ducommun B, Brambilla P, Felix MA, Franza Jr BR, Karsenti E and Draetta G. (1991). *EMBO J.*, **10**, 3311–3319.
el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell*, **75**, 817–825.
Elledge SJ. (1996). *Science*, **274**, 1664–1672.
Fornace Jr AJ, Alamo Jr I and Hollander MC. (1988). *Proc Natl Acad Sci USA*, **85**, 8800–8804.
Fornace Jr AJ, Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fargnoli J and Holbrook NJ. (1989). *Mol. Cell. Biol.*, **9**, 4196–4203.
Hall PA, Kearsey JM, Coates PJ, Norman DG, Warbrick E and Cox LS. (1995). *Oncogene*, **10**, 2427–2433.
Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ. (1993). *Cell*, **75**, 805–816.
Hartwell LH and Kastan MB. (1994). *Science*, **266**, 1821–1828.
Hartwell LH and Weinert TA. (1989). *Science*, **246**, 629–634.
Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW and Vogelstein B. (1997). *Mol. Cell*, **1**, 3–11.
Hollander MC, Sheikh MS, Bulavin DV, Lundgren K, Augeri-Henmueller L, Shehee R, Molinaro TA, Kim KE, Tolosa E, Ashwell JD, Rosenberg MP, Zhan Q, Fernandez-Salguero PM, Morgan WF, Deng CX and Fornace Jr AJ. (1999). *Nat. Genet.*, **23**, 176–184.
Jin S, Antinore MJ, Lung FD, Dong X, Zhao H, Fan F, Colchagie AB, Blanck P, Roller PP, Fornace Jr AJ and Zhan Q. (2000). *J. Biol. Chem.*, **275**, 16602–16608.
Kastan MB, Onyekwere O, Sidransky D, Vogelstein B and Craig RW. (1991). *Cancer Res.*, **51**, 6304–6311.
Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace Jr AJ. (1992). *Cell*, **71**, 587–597.
Kearsey JM, Coates PJ, Prescott AR, Warbrick E and Hall PA. (1995). *Oncogene*, **11**, 1675–1683.
Kohn KW, Jackman J and O'Connor PM. (1994). *J Cell Biochem*, **54**, 440–452.
Muschel RJ, Zhang HB, Iliakis G and McKenna WG. (1991). *Cancer Res.*, **51**, 5113–5117.
Muschel RJ, Zhang HB, Iliakis G and McKenna WG. (1992). *Radiat Res*, **132**, 153–157.
O'Connor PM. (1997). *Cancer Surv.*, **29**, 151–182.
O'Connor PM and Fan S. (1996). *Prog. Cell Cycle Res.*, **2**, 165–173.
O'Connor PM and Kohn KW. (1992). *Semin. Cancer Biol.*, **3**, 409–416.
Papathanasiou MA, Kerr NC, Robbins JH, McBride OW, Alamo Jr I, Barrett SF, Hickson ID and Fornace Jr AJ. (1991). *Mol. Cell. Biol.*, **11**, 1009–1016.
Paulovich AG, Toczyski DP and Hartwell LH. (1997). *Cell*, **88**, 315–321.
Sherr CJ and Roberts JM. (1995). *Genes Dev.*, **9**, 1149–1163.
Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM and Fornace Jr AJ. (1994). *Science*, **266**, 1376–1380.
Soria JC, Jang SJ, Khuri FR, Hassan K, Liu D, Hong WK and Mao L. (2000). *Cancer Res.*, **60**, 4000–4004.
Stewart N, Hicks GG, Paraskevas F and Mowat M. (1995). *Oncogene*, **10**, 109–115.
Takekawa M and Saito H. (1998). *Cell*, **95**, 521–530.
Thompson DA, Belinsky G, Chang TH, Jones DL, Schlegel R and Munger K. (1997). *Oncogene*, **15**, 3025–3035.
Toyoshima F, Moriguchi T, Wada A, Fukuda M and Nishida E. (1998). *EMBO J.*, **17**, 2728–2735.
Waldman T, Kinzler KW and Vogelstein B. (1995). *Cancer Res.*, **55**, 5187–5190.
Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace Jr AJ and Harris CC. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 3706–3711.
Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R and Beach D. (1993). *Nature*, **366**, 701–704.
Yu M, Zhan Q and Finn OJ. (2002). *Mol. Immunology*, **38**, 981–987.
Zhan Q, Antinore MJ, Wang XW, Carrier F, Smith ML, Harris CC and Fornace Jr AJ. (1999). *Oncogene*, **18**, 2892–2900.
Zhan Q, Bae I, Kastan MB and Fornace Jr AJ. (1994a). *Cancer Res.*, **54**, 2755–2760.
Zhan Q, Chen IT, Antinore MJ and Fornace Jr AJ. (1998). *Mol. Cell Biol.*, **18**, 2768–2778.

Zhan Q, Fan S, Smith ML, Bae I, Yu K, Alamo Jr I, O'Connor PM and Fornace Jr AJ. (1996). *DNA Cell Biol.*, **15,** 805–815.

Zhan Q, Lord KA, Alamo Jr I, Hollander MC, Carrier F, Ron D, Kohn KW, Hoffman B, Liebermann DA and Fornace Jr AJ. (1994b). *Mol. Cell Biol.*, **14,** 2361–2371.

Zhang H, Hannon GJ, Casso D and Beach D. (1994). *Cold Spring Harb. Symp. Quant. Biol.*, **59,** 21–29.

Zhao H, Jin S, Antinore MJ, Lung FD, Fan F, Blanck P, Roller P, Fornace Jr AJ and Zhan Q. (2000). *Exp. Cell. Res.*, **258,** 92–100.

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Appendix 2

BRCA1 activation of the *GADD45* promoter

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Breast cancer susceptibility gene BRCA1 has been implicated in the control of gene regulation and such regulated genes are thought to mediate the biological role of BRCA1. Overexpression of BRCA1 induces *GADD45*, a p53-regulated and stress-inducible gene. However, the molecular mechanism by which BRCA1 induces the expression *GADD45* remains unclear. In this report, we have shown that the *GADD45* promoter is strongly activated following expression of wild-type BRCA1. In contrast, both the tumor-derived BRCA1 mutants (p1749R and Y1853insA) and truncated BRCA1 mutant protein (Δ500–1863 BRCA1), which lack transactivation activity, were unable to activate the *GADD45* promoter, indicating that the BRCA1-mediated activation of the *GADD45* promoter requires normal transcriptional properties of BRCA1. BRCA1 did not induce the c-Jun and c-fos promoters, which rules out a general effect of BRCA1 on other immediate-responsive genes. Expression of the human papillomavirus E6 and the dominant-negative mutant p53 proteins had no effect on the induction of the *GADD45* promoter by BRCA1, suggesting that activation of the *GADD45* promoter by BRCA1 is independent of cellular p53 function. With the 5′-deletion analysis, the BRCA1-responsive element of the *GADD45* promoter was mapped at the region from −121 to −75. Disruption of this region resulted in the abrogation of BRCA1 activation of the *GADD45* promoter. Taken together, these results demonstrate that the mechanism by which BRCA1 induces *GADD45* is mainly through the transactivation of the *GADD45* promoter, further demonstrating the evidence that *GADD45* acts as one of the BRCA1-regulated genes. *Oncogene* (2000) **19,** 4050–4057.

Keywords: *GADD45*; BRCA1; p53; gene regulation

Introduction

Breast cancer is the most frequent malignancy in women. More than half of the hereditary breast cancer can be attributed to mutations in the breast cancer susceptibility gene BRCA1, (Easton *et al.*, 1995; Ford and Easton, 1995; Miki *et al.*, 1994). Human BRCA1 encodes an 1863 amino acid nuclear phosphoprotein (Miki *et al.*, 1994) that is expressed in a variety of human tissues (Marquis *et al.*, 1995). BRCA1 levels are cell cycle-dependent, with peak levels at the G1/S border and low levels in late S or G2 (Rajan *et al.*, 1996; Vaughn *et al.*, 1996). Normally, BRCA1 associates and colocalizes with Rad51, the human homologue of the *Escherichia coli* RecA protein, and may be involved in the process of DNA recombination. Following exposure to DNA damage, BRCA1 becomes hyperphosphorylated and then dynamically redistributes to the complexes containing proliferating cell nuclear antigen (PCNA), suggesting that BRCA1 may participate in a replication checkpoint response (Scully *et al.*, 1997). It has also been found that BRCA1 plays an important role in the transcription-coupled repair of DNA damage (Gowen *et al.*, 1998). Several lines of evidence demonstrate that BRCA1 plays an important role in the control of cell cycle progression. Introduction of the COOH terminus of BRCA1 (residues 1293–1863), which behaves in a dominant-negative manner, into nontumorigenic human breast epithelial cells can cause a reduction in the doubling time and abrogation of the G2-M block induced by the spindle inhibitor colchicine (Larson *et al.*, 1997). Additionally, Murine embryos carrying a BRCA1 null mutation exhibit hypersensitivity to DNA damage and chromosomal abnormalities (Shen *et al.*, 1998). Disruption of endogenous BRCA1 protein through introducing antisense oligonucleotides to BRCA1 mRNA accelerated the growth of normal and malignant mammary cells (Thompson *et al.*, 1995). Similarly, NIH3T3 cells with a stable expression of antisense BRCA1 mRNA displayed an accelerated growth rate and tumorigenesis in nude mice (Rao *et al.*, 1996). In addition, introduction of wild-type BRCA1 into cells resulted in the growth suppression in both breast and ovarian tumor lines (Holt *et al.*, 1996).

A number of observations have implicated BRCA1 in the control of transcriptional regulation. BRCA1 has an N-terminal ring finger domain and a C-terminal transcription activation domain that activates transcription when linked to a DNA-binding domain (Chapman and Verma, 1996). It has been shown that BRCA1 may interact with RNA helicase (Anderson *et al.*, 1998), and the transcriptional regulators p53 (Ouchi *et al.*, 1998; Zhang *et al.*, 1998) and c-Myc (Wang *et al.*, 1998). Transient transfection of BRCA1 expression vector results in transcriptional activation of the p21 promoter via both p53-dependent and -independent pathways (Somasundaram *et al.*, 1997). Recent finding by Harkin *et al.* has demonstrated that overexpression of BRCA1 induces endogenous *GADD45* mRNA, leads to the JNK/SAP-dependent apoptosis (Harkin *et al.*, 1999). This evidence suggests that *GADD45* should be one of BRCA1-targeted genes

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and might play a role in mediating BRCA1's biological functions. However, the mechanism by which BRCA1 induces *GADD45* mRNA remains unclear.

GADD45 is a genotoxic stress-responsive gene and is induced by a variety of DNA damaging agents, such as UV radiation, methyl methanesulfonate (MMS) and ionizing radiation (IR) (Fornace *et al.*, 1988; Papathanasiou *et al.*, 1991). In response to IR, *GADD45* induction is regulated by the tumor suppressor p53 probably through the p53-binding motif located at the third intron of the *GADD45* gene (Kastan *et al.*, 1992; Zhan *et al.*, 1994a). Expression of Gadd45 protein suppresses cell growth in multiple tumor lines (Zhan *et al.*, 1994b). Gadd45 protein has been found to interact with PCNA (Smith *et al.*, 1994), p21 (Kearsey *et al.*, 1995), Cdc2 (Zhan *et al.*, 1999), core histone (Carrier *et al.*, 1999) and MTK/MEKK4 (Takekawa and Saito, 1998), indicating that *GADD45* may be involved in multiple important cellular events. Most recently, *GADD45* has been shown to play a role in the control of cell cycle G2-M checkpoint following certain DNA damaging treatments (Wang *et al.*, 1999).

In the present study, we investigated the mechanism of how BRCA1 induces expression of *GADD45* mRNA. When the *GADD45* promoter was linked to a chloramphenical acetyltransferase (CAT) reporter gene and cotransfected with the BRCA1 expression vector into several human cell lines, we have found that *GADD45* promoter, as measured by CAT activity, was strongly activated following BRCA1 expression. In contrast, BRCA1 does not activate c-*Jun* and c-*fos* promoters, which rule out a general effect of BRCA1 on all the immediate-responsive genes. Importantly, the induction of the *GADD45* promoter by BRCA1 was shown to require the transcriptional properties of BRCA1 since either mutations or deletion of the transcriptional domain in BRCA1 protein abrogated the BRCA1 activation of the *GADD45* promoter. The induction of *GADD45* promoter by BRCA1 was not affected by the HPVE6 and the dominant-negative mutant p53, suggesting the BRCA1-mediated activation of the *GADD45* promoter is p53-independent. Furthermore, using the deletion analysis, the BRCA1 responsive-elements of the *GADD45* promoter were mapped at the promoter region from -121 to -75.

Results

BRCA1 strongly activates GADD45 *promoter*

To explore the mechanism(s) of how BRCA1 transcriptionally up-regulates *GADD45* expression, we tested the effect of BRCA1 on activation of the *GADD45* promoter. In the initial experiments, two *GADD45* promoter reporter constructs (pHG45-CAT1 and pHG45-CAT2) were co-transfected with pCR3-BRCA1 (BRCA1) expression vector into human breast carcinoma MCF-7 or human colorectal carcinoma HCT116 cells and followed by the employment of CAT assay. As a control, pCMV-neo vector was included. As shown in Figure 1a, two *GADD45* promoter CAT reporter constructs (pHG45-CAT1 that contains the *GADD45* promoter region from -2256 to $+144$ and pHG45-CAT2 that contains the *GADD45* promoter from -909 to $+144$) were strongly activated following expression of BRCA1 in both cell lines. In contrast, pCMV-neo did not exhibit activating effect on the *GADD45* promoter. To determine transfection efficiency, 4 μg of GFP (green fluorescence protein) expression vector was co-transfected with each tested plasmid. The expression of GFP protein indicated that transfection efficiency is similar among different samples and the variations were seen less than 20%. To confirm the activation of the *GADD45* promoter by BRCA1 is specifically due to the BRCA1 transactivation activity, several constructs expressing the tumor-derived BRCA1 mutants (P1749R and Y1853insA) and truncated mutant (Δ500–1863 BRCA1) were employed. Those mutants are transactivation-deficient. As a positive control, the pWW-CAT, a p21 promoter CAT reporter construct, was included in the experiments. In Figure 1b, mutants of BRCA1 lacking normal transactivation domain were deficient in activating both the *GADD45* and p21 promoters, indicating that the intact transcriptional capability of BRCA1 is required for the induction of the *GADD45* promoter.

It should be noted here that there is a discrepancy between these results and the report by Harkin *et al.* (1999). In Harkin's report, there was no activation of the *GADD45* proximal promoter (from -994 to $+1$) by expression of BRCA1. Therefore, we constructed two GADD45 promoter CAT reporters spanning -994 to $+1$ and -909 to $+1$, and tested them in four different cell lines including MCF-7, H1299, HCT116 and Hela. Similarly, the proximal promoter CAT reporters were strongly activated by BRCA1 and results were shown in Figure 1c. In agreement with Harkin's observation that intron 3 of *GADD45* was BRCA1-responsive, we found REX5, which contains five copies of a p53 consensus that is identical to *GADD45* intron 3, was activated following BRCA1 expression. In our experiments, the *GADD45* promoter seems to be more potent in mediating BRCA1-dependent transcriptional activation compared to the intron 3 element of GADD45 (Figure 1b).

We next examined whether introduction of BRCA1 expression vector into human cells induces the expression of endogenous *GADD45* mRNA. To do that, BRCA1 and pCI Tac expression vectors were co-introduced into MCF-7. Thirty-six hours later, Tac-positive cells were isolated for RNA analysis. As shown in Figure 1d, using RNase protection assay, the levels of endogenous *GADD45* mRNA were found to evidently elevate in MCF-7 cells. Taken together, these results indicate that induction of *GADD45* mRNA by BRCA1 is mediated through the transcriptional activation of *GADD45* promoter following BRCA1 expression and activation of the *GADD45* promoter depends on normal transcriptional function of BRCA1.

BRCA1 activates GADD45 *promoter in a p53-independent manner*

In cellular response to DNA damaging agents, *GADD45* induction by ionizing radiation (IR) strictly depends on normal cellular p53 function (Kastan *et al.*, 1992; Zhan *et al.*, 1994a). However, following exposure to certain non-IR agents such as UV irradiation and MMS, p53 is not required for the *GADD45* induction

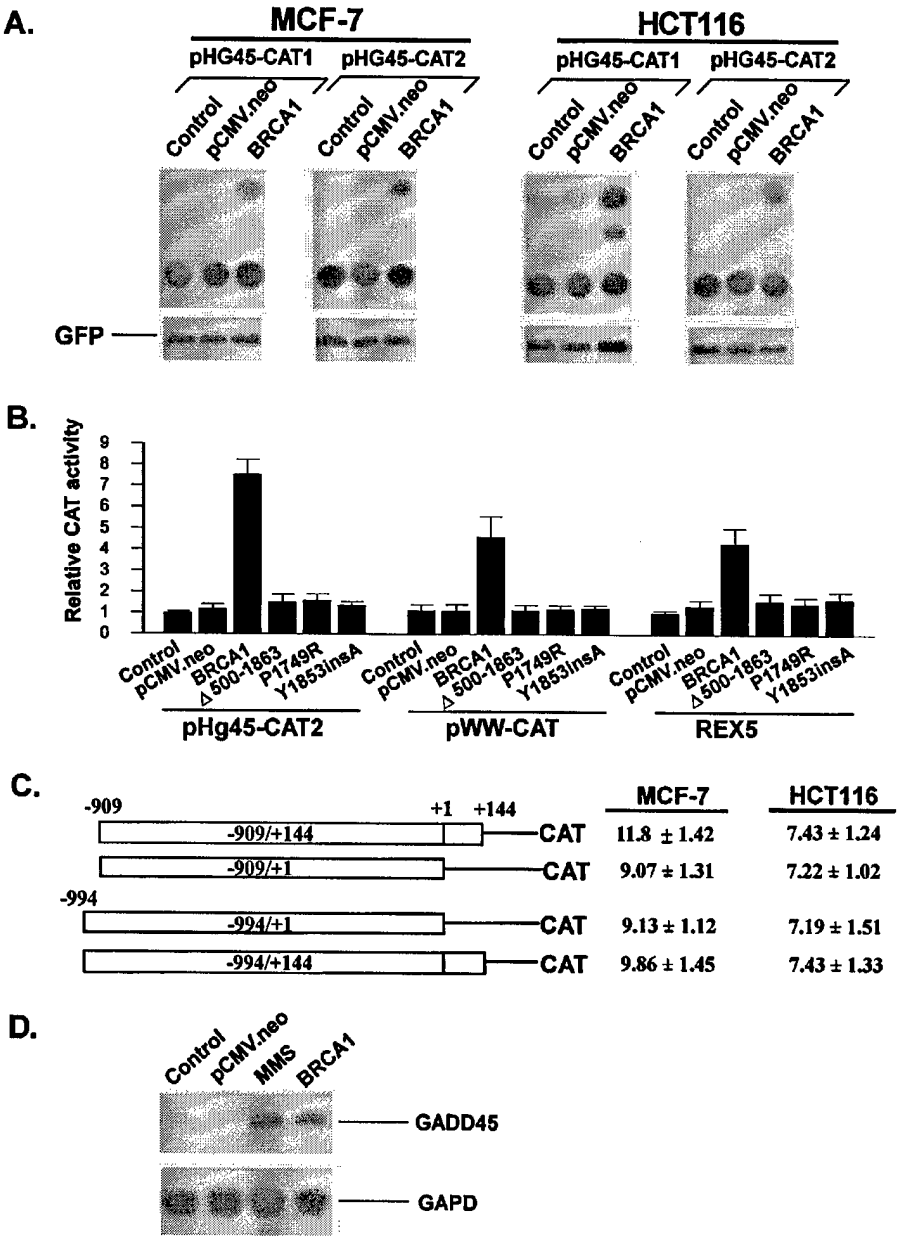

Figure 1 Activation of the *GADD45* promoter following expression of BRCA1. (**a**) 4 μg of the *GADD45* promoter CAT reporter constructs (pHG45-CAT1 and pHG45-CAT2) were cotransfected with 4 μg of either pCR3.BRCA1 (BRCA1) and pCMV·neo expression vectors into MCF-7 and HCT116 cells via the calcium phosphate method. Forty-eight hours later, cells were collected for the CAT assay (see Materials and methods). To determine transfection efficiency, 4 μg of GFP (green fluorescence protein) expression vector was co-transfected with each tested plasmid and the expression of GFP protein was detected as the internal control of transfection. (**b**) pHG45-CAT2, pWW-CAT (p21 promoter reporter construct) and REX5 (a reporter construct that contains five copies of a p53 consensus that is identical to *GADD45* intron 3) were transfected with the BRCA1 mutants of lacking transactivation activity into HCT116 cells. The CAT assays were performed as in (**a**). (**c**) The activating effect of BRCA1 on the *GADD45* proximal promoter. pHG45-CAT23 (+1 to −909 bp upstream of transcriptional start site) and pHG45-CAT30 (containing the region from +1 to 994) were co-transfected with the BRCA1 expression vector into MCF-7 and HCT116 cells. The CAT assays were carried out as in (**a**). (**d**) MCF-7 cells were transfected with 1 μg of pcI Tac and either 10 μg of pCMV·neo (lane 2) or pCR3·BRCA1 (lane 4). The Tac-positive cells were isolated. 10 μg of whole-cell RNA were hybridized with the human *GADD45* and *GAPD* RBI-probes by RNase protection assay (see Materials and methods). As a positive control, an RNA sample from MCF-7 cells treated with MMS at 100 μg/ml for 4 h was included (lane 3)

but p53 can greatly contribute to those cellular responses (Zhan *et al*., 1996). To test whether activation of the *GADD45* promoter by BRCA1 involves normal p53 function, several human lines with known p53 status were utilized. pHG45-CAT2 plasmid was transfected into MCF-7 that contains wt p53 and its isogenic line, MCF7-E6 where HPV16-E6 is stably integrated and much of the p53's biological function has been abrogated. As shown in Figure 2a, BRCA1 was shown to strongly activate pHG45-CAT2

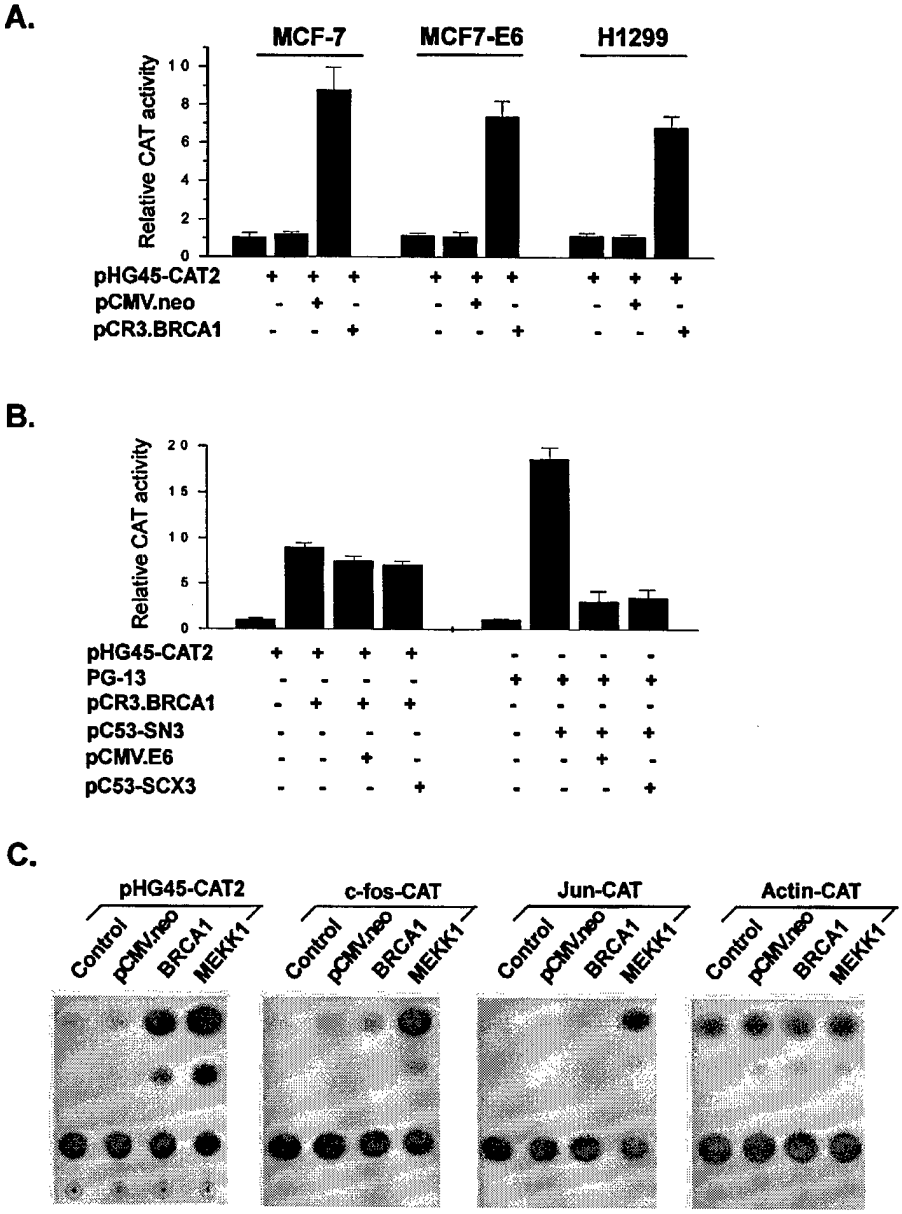

Figure 2 Induction of the *GADD45* promoter by BRCA1 is p53-independent. (**a**) pHG45-CAT2 was cotransfected with pCR3·BRCA1 into MCF-7, MCF7-E6 (with stable integration of HPVE6 gene) and H1299 cells, which has deletions of the p53 gene. The CAT assay was performed as in Figure 1. (**b**) pHG45-CTA2 or PG-13 (p53 reporter construct) were cotransfected with the indicated plasmids into MCF-7 cells and the CAT assays were followed 40 h post-transfection. (**c**) The c-*Jun*, c-*fos*, actin and MEKK1 (an activator of JNK and p38 kinase) promoter CAT reporter constructs were cotransfected with the pCR3.BRCA1 expression vector into MCF-7 cells as in (**a**). The CAT activities were measured as described in Materials and methods

in both MCF-7 and MCF7-E6 lines. Expression of E6 protein did not attenuate the induced-CAT activity of pHG45-CAT2 following BRCA1 expression. In support of these findings, pHG45-CAT2 was also strongly responsive to BRCA1 expression in human H1299 cells, which have a deletion of endogenous p53. Similarly, when the E6 or the dominant-negative mutant p53 expression vectors were transiently co-transfected into MCF-7 cells with pHG45-CAT2, induction by BRCA1 remained unchanged (see Figure 2b). In contrast, activation of PG-13, a p53 reporter construct, by p53 was abrogated following expression of E6 and mutant p53 proteins. Therefore, the results indicate that activation of *GADD45* promoter by BRCA1 does not require normal cellular p53 function.

To further determine the transactivation of the *GADD45* promoter by BRCA1 is a specific effect rather than a general effect on the stress-responsive genes, c-*Jun* and c-*fos* promoter CAT reporter constructs were cotransfected with BRCA1 expression vector and the CAT assay was followed to measure CAT activity. In Figure 2c, overexpression of BRCA1 protein did not activate the c-*Jun* and c-*fos* promoter although these two genes are induced in response to a

variety of genotoxic stress agents that also activate *GADD45* expression. In contrast, the c-*fos*-CAT and *Jun*-CAT reporter constructs were activated by protein expression of MEKK1, an activator of JNK and p38 kinase. Again, BRCA1 had no effect on the actin promoter. Therefore, BRCA1 expression is not generally associated with the activation of other stress-induced gene promoters, such as c-*Jun* and c-*fos*.

Mapping of BRCA1-responsive region in the GADD45 *promoter*

In order to localize the control elements involved in activation of the *GADD45* promoter by BRCA1, analysis of the human *GADD45* promoters with different 5′-deletions was undertaken. As shown in Figure 3a, the longest promoter, pHG45-CAT1, was strongly responsive to expression of BRCA1. With progressive 5′ deletions, induction of the *GADD45* promoter did not change substantially until the last construct, pHG45-CAT3, which extended 5′ to −74 relative to the transcription start site. With this minimal promoter, expression of BRCA1 had little effect. Since the pHG45-CAT11, which contains the promoter region spanning from −121 to +144, still showed strong induction by BRCA1, the region between −121 and −75 may contain the major controlling elements required for responsiveness to the expression of BRCA1. Interestingly, all *GADD45* promoter deletion constructs presented similar patterns of induction by BRCA1 in both MCF7 and H1299 cell lines. To further determine if the region between −121 and −75 was required for induction of the *GADD45* promoter by BRCA1, similar experiments were carried out with a reporter construct containing a deletion from −121 to −75. When this region was deleted in pHG45-CAT2ma, BRCA1-induction of the *GADD45* promoter was markedly reduced and more than 70% of the effect was lost compared to that of pHG45-CAT2 (Figure 3b).

We next used a labeled double-strand oligonucleotide corresponding to the sequence between −121 and −71 of the *GADD45* promoter for EMSAs. Although one specific prominent DNA-protein complex was observed, we were unable to demonstrate BRCA1 protein directly binding to the promoter using a supershift assay (results not shown). These results suggest that BRCA1 regulation of the GADD45 promoter might not involve a direct binding of BRCA1 protein to the promoter.

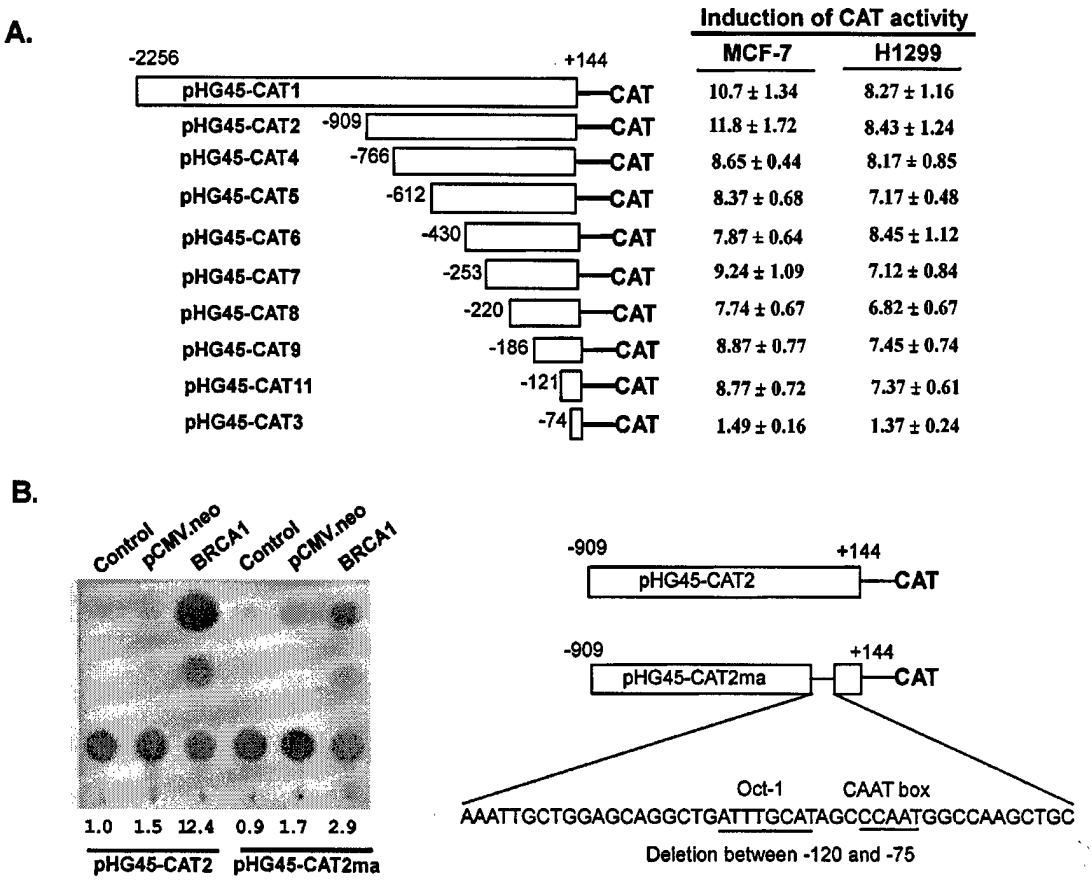

Figure 3 Mapping of the promoter region that contains the BRCA1-responsive elements. (a) Localization of the BRCA1-responsive element in the *GADD45* promoter. 4 μg of the CAT reporter constructs containing the indicated regions of the *GADD45* promoter were cotransfected with pCR·BRCA1 into MCF-7 and H1299 cell lines. The CAT assay was carried out as in Figure 1a. (b) pHG45-CAT2ma, in which the promoter sequence from −121 to −75 was deleted, was cotransfected with the BRCA1 expression vector and the CAT activities were measured as in Figure 1a. The assays were at least conducted three times but only the representative experiment is shown here

Discussion

In this report, we have analysed the effect of BRCA1 on activation of the human *GADD45* promoter in several human cell lines. We found that expression of wild type BRCA1 strongly activates the human *GADD45* promoter, while the BRCA1 mutants of lacking transcriptional activity failed to induce the *GADD45* promoter. Activation of *GADD45* by BRCA1 is in a p53-independent manner and this activation is not due to the BRCA1-induced general effect on the stress-inducible genes since BRCA1 does not activate c-*jun* and c-*fos* promoters. Using the 5′-deletion analyses, the region of the *GADD45* promoter between −121 to −75 was found to associate with the BRCA1-regulatory elements. Deletion of this region substantially abrogated the BRCA1-activation of the *GADD45* promoter. Therefore, our findings have demonstrated that the mechanism by which BRCA1 induces *GADD45* mRNA is through the transactivation of the *GADD45* promoter. Theses evidence further support the observation that *GADD45* may act as one of the BRCA1-downstream genes.

Inherited mutations in the BRCA1 gene are associated with high risk of developing breast cancer (Easton *et al.*, 1995; Ford and Easton, 1995; Miki *et al.*, 1994). A number of studies have linked BRCA1 to tumor growth suppression (Rao *et al.*, 1996), apoptosis, DNA repair, transcriptional regulation and the control of cell cycle progression (Anderson *et al.*, 1998; Chapman and Verma, 1996; Gowen *et al.*, 1998; Scully *et al.*, 1997; Somasundaram *et al.*, 1997). Inactivation of BRCA1 has been found to correlate with genomic instability (Shen *et al.*, 1998). Much effort has been put on exploring the mechanism(s) of how BRCA1 participates in these important biological events. Recent report by Harkin *et al.* (1999) demonstrated that expression of BRCA1 induces *GADD45* mRNA. This indicated that the biological roles for BRCA1 as a tumor suppressor might be mediated at least in part through its downstream genes, such as p21 and *GADD45*. It has been shown that *GADD45* can be induced by a variety of DNA damaging agents and is regulated by the tumor suppressor p53. A number of studies have suggested that *GADD45* be involved in the processes of apoptosis, DNA repair and signaling pathway (Smith *et al.*, 1994, 1996; Takekawa & Saito, 1998). Recently, Gadd45 protein has implicated in the control of cell cycle G2-M checkpoint via the inhibition of Cdc2/cyclin B1 kinase activity (Wang *et al.*, 1999; Zhan *et al.*, 1999). Therefore, it is postulated that *GADD45* might be also able to mediate the role of BRCA1 in the control of cell cycle progression, whose disruption leads to genomic instability and tumorigenesis.

In the initial promoter-mapping work, the promoters employed in our CAT reporter constructs extend 3′ to +144 relative to the transcription start site. However, in Harkin's report (Harkin *et al.*, 1999), the authors did not observe activation of the *GADD45* promoter by BRCA1 when using the promoter region from −994 to +1. For this reason, we further constructed two more promoter reporters, which contain the regions from −994 to +1 and −994 to +144. Both reporters showed strong activation following the expression of BRCA1 in four different human cell lines. This could be due to the difference in cell types tested because we also did not observe significant activation of the GADD45 promoter by BRCA1 in U2OS cells, which were used in Harkin's report (results not shown). Therefore, it can be assumed that there might be certain inhibitory factors existing in U2OS cells or lack of some mediating-factors, which are required for BRCA1 transactivation, in those cells. We have also identified that the 5′ region from −121 to −75 is required for the BRCA1-mediated activation of the *GADD45* promoter. Deletion of this region remarkably reduced the induction of *GADD45* by BRCA1. Interestingly, this region has been recently found to contain the important DNA-damage responsive elements (paper in preparation). Therefore, the results indicate that the region between −121 and −75 plays a crucial role in the regulation of the *GADD45* gene in response to either DNA damage or growth arrest signals. Analysis of DNA sequence shows that there are two known transcriptional binding sites (Oct-1 and CAAT box) in this region. To our knowledge there is currently no report to demonstrate that BRCA1 can physically associate with the proteins binding to these two sites. The future study will be focused on the dissection of this region in order to characterize the specific responsive elements regulated by BRCA1.

The precise molecular and biochemical mechanisms of how BRCA1 activates the *GADD45* promoter require further investigation. At the current time, BRCA1 has not been well characterized as a DNA-binding transcription factor. In our EMSA experiments, we were unable to demonstrate BRCA1 directly binding to the *GADD45*, but we were able to detect Oct-1 protein binding to this region in a supershift assay (results not shown). Therefore, it can be speculated that BRCA1 might activate the *GADD45* promoter probably through its interaction with another protein(s) that directly binds to the *GADD45* promoter. The interactions between the BRCA1 and the third protein(s) might also involve more complex signal transduction cascade. The similar observation has been obtained in our previous report. In it, p53 has been found to participate in the transcriptional induction of the *GADD45* promoter through its interaction with WT1 protein that is a transcription factor and directly binds to *GADD45* promoter (Zhan *et al.*, 1998). In addition, the post-translational modification, such as phophorylation and acetylation, of BRCA1 and its interactions with transcriptional co-activators might also be involved in the regulation of *GADD45* promoter. Taken all together, this study has further confirmed the BRCA1-*GADD45* pathway and provided the insight into the understanding of how BRCA1 regulates it downstream genes as well.

Materials and methods

Plasmid clones

The following plasmid constructs were used: pHG45-CAT1, constructed by inserting the *Sal*I-*Sma*I fragment of *GADD45* promoter spanning −2256 to +144 relative to the transcription start site into pCAT-Basic (promega); pHG45-CAT2, similarly constructed by inserting the *Hind*III-*Sma*I fragment

of *GADD45* promoter from −909 to +144 into pCAT-Basic (Zhan *et al.*, 1998). pHG-CAT23 was constructed by inserting the fragment of the *GADD45* promoter from 994 to +1 into pCAT-Basic. Dr Weber provided pCR3-BRCA1, a construct expressing wt human BRCA1 protein, and BRCA1 mutants including pCR3-BRCA1$_{1749R}$, pCR3-BRCA1$_{1853insA}$ and pCR3-BRCA1$_{\Delta 500-1863}$ (Somasundaram *et al.*, 1997; Thakur *et al.*, 1997). Dr Vogelstein provided pC53-SCX3, which expresses a dominant-negative mutant p53 protein containing a substitution of Ala for Val-143 and PG-13, which contains 13 repeats of a p53-binding site inserted 5′ to the polyomavirus promoter linked to a CAT report construct (Kern *et al.*, 1992). pCMV-E6 expresses human HPVE6 protein and has been shown to block p53 function (Kessis *et al.*, 1993). c-fos-CAT was described as previously (Zhan *et al.*, 1998). Dr Ashwell provided Tac the Tac expression vector pCI.

Cell lines, tissue culture and transfection

The human breast carcinoma MCF-7 line and its subline MCF7-E6, the human lung carcinoma line H1299 and the human colorectal carcinoma line HCT116 were grown in F12 medium supplemented with 10% fetal bovine serum. The cells were transfected by the calcium phosphate (Zhan *et al.*, 1993). In these experiments, 4 μg of the *GADD45* promoter reporter constructs and 4 μg of indicated expression vectors were cotransfected into human cells. Forty hours later, cells were collected for the CAT assay. In addition, 4 μg of pCMV-GFP plasmid (which contains green fluorescence protein) was included in each experiment. After transfection, expression of GFP protein is detected by Western blotting assay to determine transfection efficiency.

For RNase protection analysis, 10 μg of either pCR3-BRCA1 or pCMV.neo were co-transfected into MCF-7 with 1.0 μg of pCI Tac, a cell surface protein expression vector, by the lipofectamine method. Forty-eight hours after transfection, cells were harvested and washed once with cold medium. Dynabeads (Dyna, Lake Success, NY, USA) coated with anti-Tac antibody were added to the cells and incubated with gentle rotation at 4°C for 2 h. The Tac-positive cells were isolated magnetically, washed once with buffer saline, and then lysed for the RNA protection assay.

CAT assay

Measurement of CAT activity was carried out as described previously (Zhan *et al.*, 1993). Cells were collected and resuspended in 0.25 M TRIS (pH 7.8). Cells were disrupted by three cycles of freeze-thaw. The equal amounts of protein were used for each CAT assay. The CAT reaction mixture was incubated at 37°C overnight and the CAT activity was determined by measuring the acetylation of ^{14}C-labeled chloramphenicol by the thin-layer chromatography. Radioactivity was measured directed with Betascope analyser. The specific CAT activity was calculated by determining the fraction of chloramphenicol that had been acetylated. The relative CAT activity was determined by normalizing the activity of the treated samples to that of the untreated sample. Each value represented the average of at least three separate determinations.

RNA isolation and RNAase protection assay

Cells were lysed in 4 M guanidine thiocynate and total cellular RNA was isolated by the acid phenol method. For RNAase protection assay, the plasmids pRibo-Hg45 and pGAPD were linearized with *Hind*III or *Bam*H1 and *in vitro* transcription was carried out at 4°C with T3 or T7 RNA polymerases. *GADD45* and *GAPD* riboprobes were labeled with [α-^{32}P]UTP. Ten μg of whole cellular RNA was hybridized with both riboprobes simultaneously in the same test tube at 53°C for 15 h and then digested with RNAase. Following protease K digestion and pheno-chloroform extraction, the samples were analysed on an 8 M urea, 5% acrylamide gel. Protected bands were visualized by autoradiography and were quantitated with a Phosphoimager analyser.

EMSA

Nuclear extracts were prepared and an electrophoretic mobility shift assay (EMSA) was carried out as described previously (Zhan *et al.*, 1998). DNA binding reactions were performed for 10 min at room temperature in a binding buffer containing 20 mM HEPES (pH 7.8), 150 mM NaCl, 1 mM dithiothreitol, 1 μg of poly(dIdC), 10% glycerol and 20 μg of nuclear protein. 4×10^4 d.p.m. of labeled probe. The probe was a 50-mer double stranded synthetic oligonucleotide containing the region spanning −121 to −71 of the *GADD45* promoter. Each strand was labeled separately and the strands were annealed, then purified by G-25 column. The samples were analysed on a 4% non-denaturing acrylamide gel (Zhan *et al.*, 1998).

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References

Anderson SF, Schlegel BP, Nakajima T, Wolpin ES and Parvin JD. (1998). *Nat. Genet.*, **19**, 254–256.
Carrier F, Georgel PT, Pourquier P, Blake M, Kontny HU, Antinore MJ, Gariboldi M, Myers TG, Weinstein JN, Pommier Y and Fornace Jr AJ. (1999). *Mol. Cell Biol.*, **19**, 1673–1685.
Chapman MS and Verma IM. (1996). *Nature*, **382**, 678–679.
Easton DF, Ford D and Bishop DT. (1995). *Am. J. Hum. Genet.*, **56**, 265–271.
Ford D and Easton DF. (1995). *Br. J. Cancer*, **72**, 805–812.
Fornace Jr AJ, Alamo Jr I and Hollander MC. (1988). *Proc. Natl. Acad. Sci. USA*, **85**, 8800–8804.
Gowen LC, Avrutskaya AV, Latour AM, Koller BH and Leadon SA. (1998). *Science*, **281**, 1009–1012.
Harkin DP, Bean JM, Miklos D, Song YH, Truong VB, Englert C, Christians FC, Ellisen LW, Maheswaran S, Oliner JD and Haber DA. (1999). *Cell*, **97**, 575–586.
Holt JT, Thompson ME, Szabo C, Robinson-Benion C, Arteaga CL, King MC and Jensen RA. (1996). *Nat. Genet.*, **12**, 298–302.
Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace Jr AJ. (1992). *Cell*, **71**, 587–597.
Kearsey JM, Coates PJ, Prescott AR, Warbrick E and Hall PA. (1995). *Oncogene*, **11**, 1675–1683.
Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW and Vogelstein B. (1992). *Science*, **256**, 827–830.
Kessis TD, Slebos RJ, Nelson WG, Kastan MB, Plunkett BS, Han SM, Lorincz AT, Hedrick L and Cho KR. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 3988–3992.
Larson JS, Tonkinson JL and Lai MT. (1997). *Cancer Res.*, **57**, 3351–3355.

Marquis ST, Rajan JV, Wynshaw-Boris A, Xu J, Yin GY, Abel KJ, Weber BL and Chodosh LA. (1995). *Nat. Genet.*, **11**, 17–26.

Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, Bell R, Rosenthal J, Hussey C, Tran T, McClure M, Frye C, Hattier T, Phelps R, Haugen-Strano A, Katcher H, Yakumo K, Gholami Z, Shaffer D, Stone S, Bayer S, Wray C, Bogden R, Dayanath P, Ward J, Tonin P, Narod S, Bristow P, Norris FH, Helvering L, Morrison P, Rosteck P, Lai M, Barrett JC, Lewis C, Neuhausen S, Cannon-Albright L, Goldgar D, Wiseman R, Kamb A and Skolnick MH. (1994). *Science*, **266**, 66–71.

Ouchi T, Monteiro AN, August A, Aaronson SA and Hanafusa H. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 2302–2306.

Papathanasiou MA, Kerr NC, Robbins JH, McBride OW, Alamo Jr I, Barrett SF, Hickson ID and Fornace Jr AJ. (1991). *Mol. Cell Biol.*, **11**, 1009–1016.

Rajan JV, Wang M, Marquis ST and Chodosh LA. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 13078–13083.

Rao VN, Shao N, Ahmad M and Reddy ES. (1996). *Oncogene*, **12**, 523–528.

Scully R, Chen J, Ochs RL, Keegan K, Hoekstra M, Feunteun J and Livingston DM. (1997). *Cell*, **90**, 425–435.

Shen SX, Weaver Z, Xu X, Li C, Weinstein M, Chen L, Guan XY, Ried T and Deng CX. (1998). *Oncogene*, **17**, 3115–3124.

Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM and Fornace Jr AJ. (1994). *Science*, **266**, 1376–1380.

Smith ML, Kontny HU, Zhan Q, Sreenath A, O'Connor PM and Fornace Jr AJ. (1996). *Oncogene*, **13**, 2255–2263.

Somasundaram K, Zhang H, Zeng YX, Houvras Y, Peng Y, Wu GS, Licht JD, Weber BL and El-Deiry WS. (1997). *Nature*, **389**, 187–190.

Takekawa M and Saito H. (1998). *Cell*, **95**, 521–530.

Thakur S, Zhang HB, Peng Y, Le H, Carroll B, Ward T, Yao J, Farid LM, Couch FJ, Wilson RB and Weber BL. (1997). *Mol. Cell Biol.*, **17**, 444–452.

Thompson ME, Jensen RA, Obermiller PS, Page DL and Holt JT. (1995). *Nat. Genet.*, **9**, 444–450.

Vaughn JP, Davis PL, Jarboe MD, Huper G, Evans AC, Wiseman RW, Berchuck A, Iglehart JD, Futreal PA.and Marks JR. (1996). *Cell. Growth Differ.*, **7**, 711–715.

Wang Q, Zhang H, Kajino K and Greene MI. (1998). *Oncogene*, **17**, 1939–1948.

Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace Jr AJ and Harris CC. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 3706–3711.

Zhan Q, Antinore MJ, Wang XW, Carrier F, Smith ML, Harris CC and Fornace Jr AJ. (1999). *Oncogene*, **18**, 2892–2900.

Zhan Q, Bae I, Kastan MB and Fornace Jr AJ. (1994a). *Cancer Res.*, **54**, 2755–2760.

Zhan Q, Carrier F and Fornace Jr AJ. (1993). *Mol. Cell Biol.*, **13**, 4242–4250.

Zhan Q, Chen IT, Antinore MJ and Fornace Jr AJ. (1998). *Mol. Cell Biol.*, **18**, 2768–2778.

Zhan Q, Fan S, Smith ML, Bae I, Yu K, Alamo Jr I, O'Connor PM and Fornace Jr AJ. (1996). *DNA Cell Biol.*, **15**, 805–815.

Zhan Q, Lord KA, Alamo Jr I, Hollander MC, Carrier F, Ron D, Kohn KW, Hoffman B, Liebermann DA and Fornace Jr AJ. (1994b). *Mol. Cell Biol.*, **14**, 2361–2371.

Zhang H, Somasundaram K, Peng Y, Tian H, Bi D, Weber BL and El-Deiry WS. (1998). *Oncogene*, **16**, 1713–1721.

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Gadd45a contributes to p53 stabilization in response to DNA damage

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p53 is an important molecule in cellular response to DNA damage. After genotoxic stress, p53 protein stabilizes transiently and accumulates in the nucleus, where it functions as a transcription factor and upregulates multiple downstream-targeted genes, including $p21^{Waf1/Cip1}$, Gadd45a and Bax. However, regulation of p53 stabilization is complex and may mainly involve post-translational modification of p53, such as phosphorylation and acetylation. Using mouse embryonic fibroblasts (MEFs) derived from Gadd45a knockouts, we found that disruption of Gadd45a greatly abolished p53 protein stabilization following UVB treatment. Phosphorylation of p53 at Ser-15 was substantially reduced in Gadd45a−/− MEFs. In addition, p53 induction by UVB was shown to be greatly abrogated in the presence of p38 kinase inhibitor, but not c-Jun N-terminal kinase (JNK) and extracellular-signal regulated kinase (ERK), suggesting that p38 protein kinase is involved in the regulation of p53 induction. Along with the findings presented above, inducible expression of Gadd45a enhanced p53 accumulation after cell exposure to UVB. Taken together, the current study demonstrates that Gadd45a, a conventional downstream gene of p53, may play a role as an upstream effector in p53 stabilization following DNA damage, and thus has defined a positive feedback signal in the activation of the p53 pathway.
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Tumor suppressor p53 gene is the most frequently mutated gene (Vogelstein and Kinzler, 1992) and has been implicated in playing an important role in maintaining genomic fidelity by controlling cell cycle checkpoints and apoptotic process following cell exposure to genotoxic stress (Kastan *et al.*, 1992; Zhan *et al.*, 1994b; Miyashita and Reed, 1995; Waldman *et al.*, 1995; White, 1996; Levine, 1997). Following DNA damage, including ionizing radiation (IR), UV radiation (UV) and a variety of DNA alkylating agents, p53 protein is stabilized and activated as a transcription factor (Kastan *et al.*, 1991; Zhan *et al.*, 1993). Activated p53 transcriptionally upregulates its downstream genes, such as $p21^{Waf1/Cip1}$ (el-Deiry *et al.*, 1993), Gadd45a (Kastan *et al.*, 1992; Zhan *et al.*, 1994a) and Bax (Zhan *et al.*, 1994b; Miyashita and Reed, 1995), whose products are able to participate directly in the control of cell cycle arrest and apoptosis. Deregulation of p53 function in the cellular response to DNA damage results in genomic instability, which is closely associated with cell transformation and tumorigenesis.

Induction of p53 protein is the key step in the activation of p53-mediated signaling pathways (Kastan *et al.*, 1992). However, the regulation of p53 stabilization after genotoxic stress is complex and it is thought to occur primarily through a post-transcriptional mechanism as there are no changes in p53 mRNA levels following cell exposure to DNA damage (Kastan *et al.*, 1991). In nonstressed circumstances, p53 is degraded via the MDM2-mediated ubiquitin pathway (Haupt *et al.*, 1997; Honda *et al.*, 1997; Fuchs *et al.*, 1998a). MDM2 is also a p53-regulated protein and able to bind to p53, promoting p53 degradation in order to limit the length of p53 function of negatively regulating cell cycle progression (Wu *et al.*, 1993; Chen *et al.*, 1994). Therefore, one of the mechanisms by which p53 becomes stabilized may be through the inhibition of MDM2 binding to p53 protein. Previous studies reported that post-translational modification of p53 protein after DNA damage is able to remove the MDM2 inhibition of p53, block MDM2-mediated degradation of p53 and in turn cause accumulation of p53 (Shieh *et al.*, 1997; Ashcroft *et al.*, 1999; Oren, 1999). There are multiple phosphorylation sites at p53 protein and these sites are subjected to phosphorylation following genotoxic stress. Although various genotoxic stresses are able to stabilize p53 protein, different types of DNA-damaging agents act on varied phosphorylation sites of p53. For example, IR treatment results in p53 phosphorylation at Ser-15, Thr-18, Ser-20 and Ser-33 (Siliciano *et al.*, 1997; Dumaz and Meek, 1999; Shieh *et al.*, 1999; Unger *et al.*, 1999). UV radiation causes p53 phosphorylation at nearly every Ser site, including Ser-15, Ser-20, Ser-33, Ser-37 and Ser-46 (Siliciano *et al.*, 1997; Sakaguchi *et al.*, 1998; Bulavin *et al.*, 1999). In addition, a number of investigations have shown that p53 phosphorylation is carried out by a variety of

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upstream kinases, such as ATM, ATR, p38, c-Jun N-terminal kinase (JNK) and Casein kinase II (Milne *et al.*, 1992; Banin *et al.*, 1998; Canman *et al.*, 1998; Canman and Lim, 1998; Bulavin *et al.*, 1999). These observations suggest that multiple signaling pathways are involved in p53 stabilization following cell exposure to various genotoxic stresses.

Gadd45a is a genotoxic stress-responsive gene and induced by a wide spectrum of DNA-damaging agents including IR, UV and many alkylating agents (Fornace *et al.*, 1988; Hollander *et al.*, 1993). Following DNA damage, Gadd45a induction is rapid, sensitive and transient (Zhan *et al.*, 1994a). Recent findings demonstrate that Gadd45a protein interacts with PCNA (Smith *et al.*, 1994), p21 (Kearsey *et al.*, 1995; Zhao *et al.*, 2000), Cdc2 (Zhan *et al.*, 1999; Jin *et al.*, 2000), MTK1/MEKK4 (Takekawa and Saito, 1998), and has been implicated in the control of cell cycle G2-M arrest (Wang *et al.*, 1999) as well as the maintenance of genomic stability. In agreement with these observations, Gadd45a knockout mice exhibit severe genomic instability and increased tumorigenesis by treatment with IR and UV radiation (Hollander *et al.*, 1999; Hildesheim *et al.*, 2002). Regulation of Gadd45a induction after DNA damage is complex and may involve both p53-dependent and -independent signaling pathways. It has been reported that Gadd45a induction by IR strictly depends on normal cellular p53 function, but Gadd45a induction by UV and MMS does not require p53 although p53 may contribute to the non-IR response of Gadd45a (Kastan *et al.*, 1992; Zhan *et al.*, 1996).

In the current study, we report that Gadd45a plays an important role in stabilization of p53 protein following UV radiation. As shown in Figure 1a, both E1A/ras-transformed normal mouse embryonic fibroblasts (MEFs, ER-1) and E1A/ras-transformed Gadd45aa−/− MEFs (ER-11) were treated with UVB radiation at the indicated doses (range from 0 to 400 J/m^2). At 8 h post-treatment, MEF cells were collected and prepared for the cellular protein as described previously. Whole-cell lysates (100 μg) were loaded onto 10% SDS–PAGE gels and measurement of p53 protein was performed with anti-mouse p53 antibody (Pharmigen, San Diego, CA, USA). In addition, actin protein was also measured as a loading control. The results in Figure 1a showed that UVB at doses of 10 or 30 J/m^2 did not significantly induce p53 in ER-1 cells, but an evident induction of p53 protein was observed in the cells treated with 100 J/m^2 of UVB. The maximal induction of p53 occurred in ER-1 cells exposed to the doses between 200 and 400 J/m^2. However, when ER-11 cells (Gadd45a−/− MEFs) were treated with UVB at the same doses (from 200 to 400 J/m^2), p53 induction was substantially weaker compared to that seen in ER-1. Next, we exposed both ER-1 and ER-11 to UVB radiation at a dose of 200 J/m^2 and detected p53 expression at the indicated time (0, 1, 2, 4, 6, 8 and 10 h). In ER-1 cells, a strong induction of p53 protein (more than sixfold) was clearly seen at the 2 h time point and maintained at a plateau till 10 h post-treatment. In contrast, ER-11 cells treated with 200 J/m^2 of UVB revealed a weaker induction of p53 protein

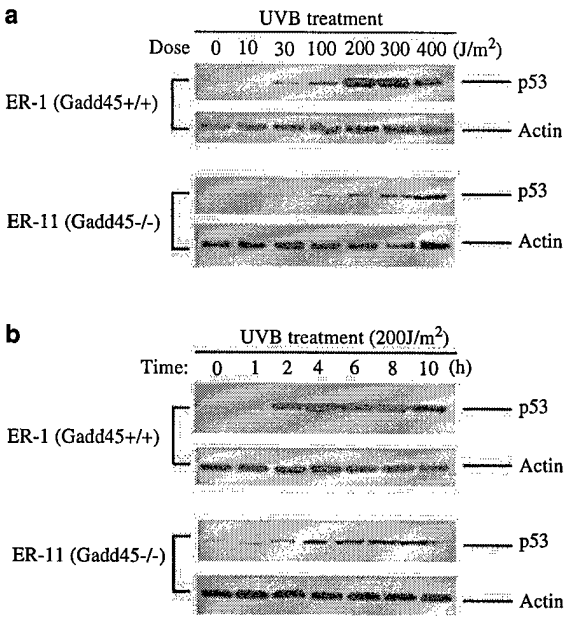

Figure 1 Attenuated induction of p53 protein in Gadd45a−/− cells treated with UVB. (**a**) Both E1A/ras-transformed normal MEFs (ER-1) and Gadd45a−/− MEFs (ER-11) were treated with UVB at different doses (range from 0 to 400 J/m^2). Cells were harvested at 8 h post-treatment and cellular proteins were prepared as described previously. Total cell protein (100 g) was loaded onto 10% SDS– PAGE gels. Following electrophoresis, the proteins were transferred to Immobilon membranes. The membranes were then blocked for 30 min in 5% milk at room temperature. Measurement of p53 protein was performed with anti-mouse p53 antibody (Santa Cruz Biotech, CA, USA). Immunoreaction was revealed using chemiluminescence detection procedure. As a loading control, detection of actin protein was included. Only visualized bands are shown; their estimated sizes were 53 kDa for p53 protein and 43 kDa for actin. All the experiments were performed at least three times. The results of the representative experiment are shown in this figure. (**b**) ER-1 and ER11 cells were exposed to UVB at a dose of 200 J/m^2 and harvested at the different time points. Detection of p53 expression was performed as described in (**a**)

(approximately twofold) at the same time points. These results indicate that Gadd45a has some role in the regulation of p53 induction after UVB treatment.

It has been well accepted that induction of p53 protein after DNA damage is mainly through a post-transcriptional mechanism. The post-translational modification of p53 at multiple phosphorylation sites greatly contributes to its stabilization following various genotoxic stresses (Shieh *et al.*, 1997; Ashcroft *et al.*, 1999; Oren, 1999). Among these phosphorylation sites, Ser-15 is critical for p53 stabilization after IR and UV (Lakin *et al.*, 1999; Tibbetts *et al.*, 1999). Therefore, we detected phosphorylation of p53 at Ser-15 using a phosphorylation-site-specific antibody (Santa Cruz Biotech, CA, USA). In Figure 2a, p53 phosphorylation at Ser-15 in ER-1 cells (Gadd45a+/+) treated with UVB was analysed. Cells were exposed to UVB at a range from 0 to 400 J/m^2 and cultures were harvested 4 h after treatment. Evidently, phosphorylation of Ser-15 in ER-1 cells was strongly induced by the exposure of the cells to UVB at doses between 200 and 400 J/m^2. Meanwhile,

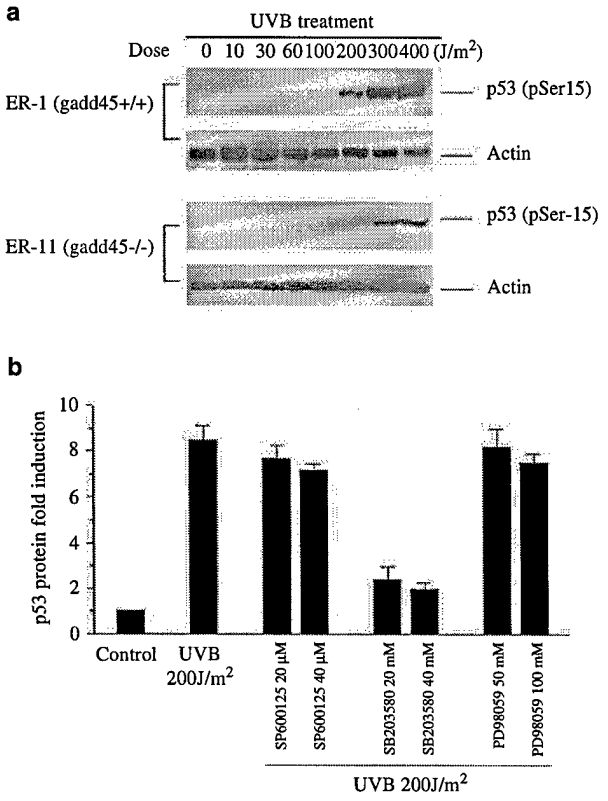

Figure 2 (**a**) Phosphorylation of p53 at Ser-15 in response to UVB treatment. Both ER-1 and ER-11 were treated with UVB radiation with different doses. Whole-cell protein was prepared and analysed for phosphorylation of p53 protein at Ser-15 using a phosphorylation-site-specific antibody. (**b**) ER-1 (E1A/ras-transformed normal MEF cells) were grown in the presence of kinase inhibitors for JNK (SP600125), p38 (SB203580) and ERK (PD98059) at the indicated concentration and treated with UVB at 200 J/m². Cellular proteins were prepared at 8 h post-treatment. Proteins (100 g) were loaded onto 10% SDS– PAGEgel for detection of cellular p53

phosphorylation of Ser-15 in ER-11 cells exhibited less than half the level of that seen in ER-1 cells, indicating that disruption of Gadd45a results in decreased phosphorylation of Ser-15 for p53 protein following DNA damage. MAP kinase pathways, which include the extracellular-signal regulated kinase (ERK), stress-activated protein kinase-1/ JNK and stress-activated protein kinase-2/p38α and β (herein called p38), are found to be involved in post-translational modification of p53 protein. All ERK, JNK and p38 kinases are reported to phosphorylate p53 protein at various sites and may play roles in the stabilization of p53 in response to genotoxic stress (Fuchs *et al.*, 1998b; Bulavin *et al.*, 1999; Sablina *et al.*, 2001; Wang and Shi, 2001). To examine which MAP kinase pathways directly contributes to UVB-induced p53 induction, we treated ER-1 cells with 200 J/m² UVB in the presence of inhibitors for ERK, JNK and p38 kinases and collected cells at 8 h post-treatment for measurement of p53 levels. As shown in Figure 2b, the addition of a specific inhibitor (SB203580) for a p38 kinase substantially reduced p53 induction by UVB. The induction of p53 in the presence of a p38 inhibitor only exhibited twofold compared with eightfold induction in ER-1 cells without an inhibitor. However, addition of either JNK kinase inhibitor (SP600125) or an ERK kinase inhibitor (PD98059) did not affect p53 induction, suggesting that activation of p38 might be required for p53 stabilization after UVB.

To further investigate the role of Gadd45a in the stabilization of p53 protein after genotoxic stress, we employed a human HCT116 Gadd45a-inducible cell line, where Gadd45a expression is controlled by the tet-off system (Jin *et al.*, 2002), and examined p53 induction in the presence of inducible expression of Gadd45a protein. In Figure 3a, HCT116 Gadd45a-inducible cells exhibited an extremely low basal level of endogenous

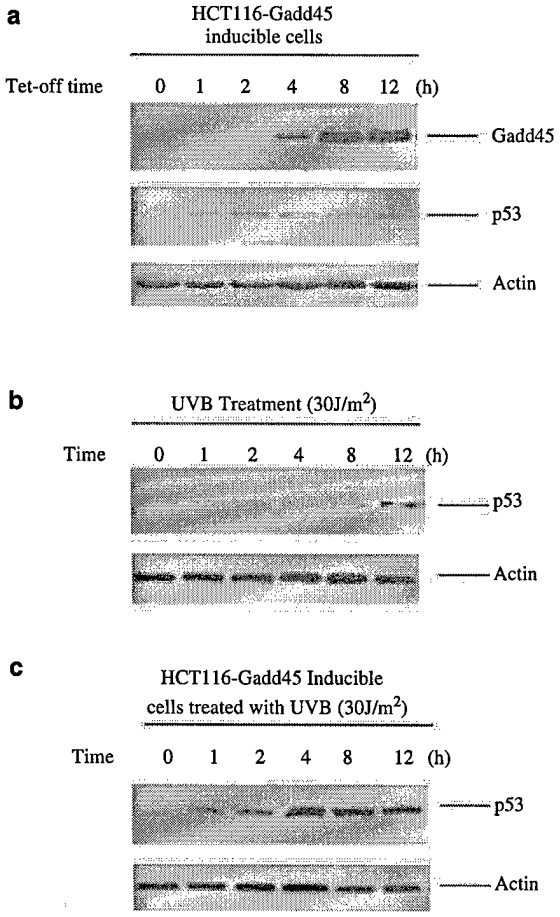

Figure 3 Expression of Gadd45a contributes to stabilization of p53 protein in response to UVB radiation. (**a**) Cellular proteins were prepared from HCT116 Gadd45a-inducible cells after the withdrawal of tetracycline at the indicated time points. Proteins (100 g) were loaded onto SDS–PAGE gel for detection of Gadd45a, p53 and actin. (**b**) HCT116 Gadd45a-inducible cells were grown in the medium containing tetracycline at 2 g/ml, and treated with a low dose of UVB (30 J/m²). Cells were collected at the indicated times for preparation of cellular protein. Proteins (100 g) were loaded onto SDS–PAGE gel for detection of Gadd45a, p53 and actin. (**c**) HCT116 Gadd45a-inducible cells were grown in medium containing tetracycline at 2 g/ml. At 6 h after the withdrawal of tetracycline, cells were exposed to UVB at a dose of 30 J/m² and then collected at the indicated time points for detection of cellular p53 protein

Gadd45a protein. After removal of tetracycline, Gadd45a protein was greatly induced. However, inducible expression of Gadd45a alone did not induce p53 protein as p53 remained at the same levels even in the presence of a 10-fold induction of Gadd45a, suggesting that Gadd45a itself is unable to cause the accumulation of p53 protein. We speculated that Gadd45a-mediated p53 induction may also require exposure of cells to genotoxic stress. To demonstrate this, we first induced Gadd45a protein in HCT116 Gadd45a-inducible cells by removing tetracycline. After 6 h, we treated those cells with UVB at 30 J/m^2, a low dose which did not induce p53 (Figure 3b). Cell cultures were collected at the indicated time points and analysed to detect p53 induction. Interestingly, UVB at a dose of 30 J/m^2 was shown to result in p53 accumulation in the presence of inducible expression of Gadd45a. These results indicate that Gadd45a sensitizes cells to a low dose of UVB in terms of p53 induction. Taken all together, the results in the current study demonstrate that Gadd45a plays a role in p53 stabilization after DNA damage.

As discussed earlier, Gadd45a is identified as one of the p53-regulated stress-inducible genes. IR induction of Gadd45a has been shown to depend on normal cellular p53 function, but Gadd45a induction by UV and other non-IR stresses does not require p53. Therefore, there are both p53-dependent and -independent pathways involved in the regulation of Gadd45a induction. Interestingly, the findings in the current study indicate that under some circumstances (cells treated with UVB), Gadd45a may be able to function as an upstream effector that is necessary for the stabilization of p53 protein since p53 induction is diminished in MEF cells with disruption of endogenous Gadd45a. Indeed, Heldesheim *et al.* recently reported that transcriptional activity of p53 following UVB treatment is greatly abrogated in MEFs derived from Gadd45a knockouts.

As to the molecular and biochemical mechanism(s) by which Gadd45a contributes to p53 stabilization, we initially examined whether Gadd45a interacts with MDM2, which associates with p53 and promotes degradation of p53 protein, and releases MDM2 inhibition of p53. In multiple experiments, we found no evidence of an interaction between MDM2 and Gadd45a (results not shown), suggesting that involvement of Gadd45a in p53 stabilization is not through direct interaction of Gadd45a with MDM2. The results in Figure 2a demonstrated that the disruption of Gadd45a affected the phosphorylation of p53 at Ser-15, which is critical for the stabilization of p53 protein. In addition, the findings in Figure 2b showed that p38 kinase, but not JNK and ERK, was involved in the stabilization of p53 protein since the employment of specific inhibitor for p38 kinase substantially abrogated p53 induction by UVB. These observations are supported by other's previous demonstration that p38 kinase is required for phosphorylation of p53 at Ser-15 in response to UV treatment (Bulavin *et al.*, 1999). Interestingly, p38 has not been reported to directly phosphorylate p53 at Ser-15 site. The effect of p38 kinase on Ser-15 is probably medicated through the cooperativity of Ser-33 and Ser-46, at which p38 is able to directly phosphorylate, because Ser-15 phosphorylation by p38 is abolished in the Ser-33/46 double mutant. Most recently, Bulavin *et al.* (2003) has found that Gadd45a protein can physically associate with p38 kinase and is necessary for p38 activation in the presence of Ha-ras . Therefore, the activation of p38 kinase by Gadd45a might be one of the mechanism(s) that are involved in Gadd45a contribution to p53 stabilization. In summary, the current study demonstrates a novel finding that Gadd45a, conventionally as one of the p53-downstream genes, is able to generate a positive 'feedback signal' that is essential for maintaining p53 protein stability and activity in response to genotoxic stress. This positive feedback loop is likely mediated through activation of p38 kinase by Gadd45a (Bulavin *et al.*, 1999).

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References

Ashcroft M, Kubbutat MH and Vousden KH. (1999). *Mol. Cell. Biol.*, **19,** 1751–1758.

Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y and Ziv Y. (1998). *Science,* **281,** 1674–1677.

Bulavin DV, Kovalsky O, Hollander MC and Fornace Jr AJ. (2003). *Mol. Cell. Biol.*, **23,** 3859–3871.

Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E and Fornace Jr AJ. (1999). *EMBO J.*, **18,** 6845–6854.

Canman CE and Lim DS. (1998). *Oncogene,* **17,** 3301–3308.

Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB and Siliciano JD. (1998). *Science,* **281,** 1677–1679.

Chen CY, Oliner JD, Zhan Q, Fornace Jr AJ, Vogelstein B and Kastan MB. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 2684–2688.

Dumaz N and Meek DW. (1999). *EMBO J.*, **18,** 7002–7010.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell,* **75,** 817–825.

Fornace Jr AJ, Alamo Jr I and Hollander MC. (1988). *Proc. Natl. Acad. Sci. USA,* **85,** 8800–8804.

Fuchs SY, Adler V, Buschmann T, Wu X and Ronai Z. (1998a). *Oncogene,* **17,** 2543–2547.

Fuchs SY, Adler V, Pincus MR and Ronai Z. (1998b). *Proc. Natl. Acad. Sci. USA,* **95,** 10541–10546.

Haupt Y, Maya R, Kazaz A and Oren M. (1997). *Nature*, **387**, 296–299.
Hildesheim J, Bulavin DV, Anver MR, Alvord WG, Hollander MC, Vardanian L and Fornace Jr AJ. (2002). *Cancer Res.*, **62**, 7305–7315.
Hollander MC, Alamo I, Jackman J, Wang MG, McBride OW and Fornace Jr AJ. (1993). *J. Biol. Chem.*, **268**, 24385–24393.
Hollander MC, Sheikh MS, Bulavin DV, Lundgren K, Augeri-Henmueller L, Shehee R, Molinaro TA, Kim KE, Tolosa E, Ashwell JD, Rosenberg MP, Zhan Q, Fernandez-Salguero PM, Morgan WF, Deng CX and Fornace Jr AJ. (1999). *Nat. Genet.*, **23**, 176–184.
Honda R, Tanaka H and Yasuda H. (1997). *FEBS Lett.*, **420**, 25–27.
Jin S, Antinore MJ, Lung FD, Dong X, Zhao H, Fan F, Colchagie AB, Blanck P, Roller PP, Fornace Jr AJ and Zhan Q. (2000). *J. Biol. Chem.*, **275**, 16602–16608.
Jin S, Tong T, Fan W, Fan F, Antinore MJ, Zhu X, Mazzacurati L, Li X, Petrik KL, Rajasekaran B, Wu M and Zhan Q. (2002). *Oncogene*, **21**, 8696–8704.
Kastan MB, Onyekwere O, Sidransky D, Vogelstein B and Craig RW. (1991). *Cancer Res.*, **51**, 6304–6311.
Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace Jr AJ. (1992). *Cell*, **71**, 587–597.
Kearsey JM, Coates PJ, Prescott AR, Warbrick E and Hall PA. (1995). *Oncogene*, **11**, 1675–1683.
Lakin ND, Hann BC and Jackson SP. (1999). *Oncogene*, **18**, 3989–3995.
Levine AJ. (1997). *Cell*, **88**, 323–331.
Milne DM, Palmer RH and Meek DW. (1992). *Nucleic Acids Res.*, **20**, 5565–5570.
Miyashita T and Reed JC. (1995). *Cell*, **80**, 293–299.
Oren M. (1999). *J. Biol. Chem.*, **274**, 36031–36034.
Sablina AA, Chumakov PM, Levine AJ and Kopnin BP. (2001). *Oncogene*, **20**, 899–909.
Sakaguchi K, Herrera JE, Saito S, Miki T, Bustin M, Vassilev A, Anderson CW and Appella E. (1998). *Genes Dev.*, **12**, 2831–2841.
Shieh SY, Ikeda M, Taya Y and Prives C. (1997). *Cell*, **91**, 325–334.
Shieh SY, Taya Y and Prives C. (1999). *EMBO J.*, **18**, 1815–1823.
Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E and Kastan MB. (1997). *Genes Dev.*, **11**, 3471–3481.
Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM and Fornace Jr AJ. (1994). *Science*, **266**, 1376–1380.
Takekawa M and Saito H. (1998). *Cell*, **95**, 521–530.
Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh SY, Taya Y, Prives C and Abraham RT. (1999). *Genes Dev.*, **13**, 152–157.
Unger T, Juven-Gershon T, Moallem E, Berger M, Vogt Sionov R, Lozano G, Oren M and Haupt Y. (1999). *EMBO J.*, **18**, 1805–1814.
Vogelstein B and Kinzler KW. (1992). *Cell*, **70**, 523–526.
Waldman T, Kinzler KW and Vogelstein B. (1995). *Cancer Res.*, **55**, 5187–5190.
Wang S and Shi X. (2001). *Carcinogenesis*, **22**, 757–762.
Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace Jr. AJ and Harris CC. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 3706–3711.
White E. (1996). *Genes Dev.*, **10**, 1–15.
Wu X, Bayle JH, Olson D and Levine AJ. (1993). *Genes Dev.*, **7**, 1126–1132.
Zhan Q, Antinore MJ, Wang XW, Carrier F, Smith ML, Harris CC and Fornace Jr AJ. (1999). *Oncogene*, **18**, 2892–2900.
Zhan Q, Bae I, Kastan MB and Fornace Jr AJ. (1994a). *Cancer Res.*, **54**, 2755–2760.
Zhan Q, Carrier F and Fornace Jr AJ. (1993). *Mol. Cell. Biol.*, **13**, 4242–4250.
Zhan Q, Fan S, Bae I, Guillouf C, Liebermann DA, O'Connor PM and Fornace Jr AJ. (1994b). *Oncogene*, **9**, 3743–3751.
Zhan Q, Fan S, Smith ML, Bae I, Yu K, Alamo Jr I, O'Connor PM and Fornace Jr AJ. (1996). *DNA Cell Biol.*, **15**, 805–815.
Zhao H, Jin S, Antinore MJ, Lung FD, Fan F, Blanck P, Roller P, Fornace Jr AJ and Zhan Q. (2000). *Exp. Cell Res.*, **258**, 92–100.

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BRCA1 Regulates *GADD45* through Its Interactions with the OCT-1 and CAAT Motifs*

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***BRCA1*, a breast and ovarian cancer susceptibility gene, has been implicated in gene regulation. Previous studies demonstrate that BRCA1 induces *GADD45*, a p53-regulated and stress-inducible gene that plays an important role in cellular response to DNA damage. However, the mechanism(s) by which BRCA1 regulates *GADD45* remains unclear. In this report, we have shown that BRCA1 activation of the *GADD45* promoter is mediated through the OCT-1 and CAAT motifs located at the *GADD45* promoter region. Site-directed mutations of both OCT-1 and CAAT motifs abrogate induction of the *GADD45* promoter by BRCA1. Both OCT-1 and CAAT motifs are able to confer BRCA1 inducibility in a nonrelated minimal promoter. Physical associations of BRCA1 protein with transcription factors Oct-1 and NF-YA, which directly bind to the OCT-1 and CAAT motifs, are established by biotin-streptavidin pull-down and co-immunoprecipitation assays. Such protein interactions are required for interaction of BRCA1 with the *GADD45* promoter because either immunodepletion of Oct-1 and NF-YA proteins or mutations in the OCT-1 and CAAT motifs disrupt BRCA1 binding to the *GADD45* promoter. These findings indicate that BRCA1 can up-regulate its targeted genes through protein-protein interactions and provide a novel mechanism by which BRCA1 participates in transcriptional regulation.**

Mutations of the breast cancer susceptibility gene, *BRCA1*, are associated with more than half the cases of hereditary breast cancer (1–3). The human *BRCA1* gene encodes a nuclear protein of 1863 amino acids and is expressed in a variety of human tissues (3, 4). Neoplastic development in *BRCA1* mutation carriers is generally accompanied by loss of the wild-type allele, suggesting BRCA1 protein may function as a tumor suppressor. A number of observations have implicated *BRCA1* in cellular response to DNA damage. BRCA1 associates and colocalizes with Rad51 protein and may be involved in DNA recombination. Following DNA damage, BRCA1 becomes hyperphosphorylated by ATM (5) and hCds1/Chk2 (6) and relocalizes to complexes containing proliferating cell nuclear antigen (7). Additionally, *BRCA1* plays an important role in the transcription-coupled repair (8) and in the control of cell cycle arrest following DNA damage (9, 10). Recently, multiple reports (11–13) have suggested that *BRCA1* might also play a role in apoptosis. Therefore, through its functions in DNA repair process, apoptosis, and cell cycle arrest, *BRCA1* plays an important role in the maintenance of genomic integrity. This is strongly supported by the demonstration that murine embryos carrying a *BRCA1* null mutation exhibit hypersensitivity to DNA damage and chromosomal abnormalities, probably due to defective G_2/M checkpoint control and improper centrosome duplication (14).

GADD45 is a DNA damage-responsive gene and is induced by a wide spectrum of genotoxic stress agents, including ionizing radiation, UV radiation, methyl methanesulfonate (MMS),[1] and medium starvation (15–17). It has been shown that induction of *GADD45* after DNA damage is mediated via both p53-dependent (18, 19) and -independent pathways (20). Expression of Gadd45 protein suppresses cell growth (21, 22). Gadd45 protein is able to associate with multiple important cellular proteins, including proliferating cell nuclear antigen (23), p21 (24, 25), Cdc2 (26), core histone (27), and MTK1/MEKK4 (28). Recent findings suggest that *GADD45* is involved in the control of cell cycle checkpoint (29) and apoptosis (28, 30). This argument is further supported by the finding that *GADD45*-null mice exhibit significant genomic instability, which is exemplified by aneuploidy, chromosomal aberrations, and gene amplification, and increased carcinogenesis following treatment with DNA damage (31). Therefore, *GADD45* appears to be an important player in maintenance of genomic stability.

Several lines of evidence support a role for *BRCA1* in transcriptional regulation. BRCA1 has an N-terminal ring finger domain and a C-terminal transcription activation domain that activates transcription when fused to a DNA-binding domain (32). It has been shown that BRCA1 interacts with transcriptional regulators, including p53 (33, 34), c-Myc (35), STAT1 (36), and estrogen receptor (37), and proteins involved in chromatin remodeling including p300/CBP (38) and RBAP46/48-HDAC (39). Expression of BRCA1 activates or suppresses expression of several important cellular proteins, such as $p21^{waf1/CIP1}$ (10) and cyclin B1 (40). Most recently, studies from our group and others (30, 40, 41) have demonstrated that BRCA1 strongly activates *GADD45* in a p53-independent manner. Activation of the *GADD45* promoter requires normal transcription-activating function of BRCA1 because the tumor-derived BRCA1 mutants (1749R and Y1853insA), which lack transcription activity, are unable to activate the *GADD45* pro-

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[1] The abbreviations used are: MMS, methyl methanesulfonate; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; GFP, green fluorescence protein; wt, wild type; mut, mutant.

moter (41). However, the molecular mechanism by which BRCA1 up-regulates *GADD45* is complex and may involve the regulatory elements located at either the third intron or the promoter region of *GADD45*. BRCA1 also represses *GADD45* expression through its interaction with ZBRK1 transcription factor (42). Despite the discrepancy of the effect of BRCA1 on *GADD45* transcription, it has been well accepted that *GADD45* is one of the BRCA1 downstream effectors and probably mediates the role of BRCA1 in maintenance of genomic stability.

The transcription factor Oct-1, a member of the POU homeodomain family, is ubiquitously expressed and binds to the AGTCAAAA consensus sequence through its DNA-binding POU domain (43). High affinity Oct-1-binding sites are found in a number of cellular promoters (44), and binding of Oct-1 factor to its consensus motif normally activates Oct-1-regulated genes (45–49). NF-Y is also a ubiquitous transcription factor consisted of three subunits, A–C. NF-Y specifically binds CAAT box motifs, which are found in 30% of eukaryotic promoters (50, 51). Recently, both Oct-1 and NF-YA, but not NF-YB and NF-YC, were found to be induced following treatment with genotoxic agents, indicating that these two transcription factors may participate in cellular response to DNA damage (52, 53).

In this article, we identify OCT-1 and CAAT as the BRCA1-regulatory elements required for BRCA1 activation of the *GADD45* promoter. Disruptions of the OCT-1 and CAAT motifs abolish activation of the *GADD45* promoter by BRCA1. Moreover, BRCA1 physically associates with Oct-1 and NF-YA transcription factors. These results characterize an important molecular mechanism by which BRCA1 regulates *GADD45*.

EXPERIMENTAL PROCEDURES

Plasmid Clones—The following *GADD45* promoter reporter constructs were used: pHG45-CAT1, pHG45-CAT2, pHG45-CAT5, pHG45-CAT7, pHG45-CAT11, pHG-CAT12, and pHG45-CAT13 (53, 54). *GADD45* promoter reporters that contain mutations in either Oct-1 or CATT box motifs (pHg45-CAT11 m1, pHg45-CAT11 m2, pHg45-CAT11 m3, pHg45-CAT11 m4, pHg45-CAT11 m5, pHg45-CAT11 m6, and pHg45-CAT11 m7) were constructed by PCR cloning as described previously (53). pCR3-BRCA1, a construct expressing wt human BRCA1 protein, was provided by B. Weber (see Ref. 10). pC53-SN3, which expresses wild-type p53 protein, was provided by B. Vogelstein (see Ref. 55). PG-CAT−107/−57 was constructed by inserting the *Hin*dIII-*Pst*I DNA fragment corresponding to −107 and −57 of the *GADD45* promoter upstream of a minimal polyomavirus early promoter linked to a CAT gene, which was derived from PG-13 CAT that was provided by Dr. B. Vogelstein. Similarly, PG-OCT-1wt or PG-OCT-1mut was constructed by cloning 5 direct repeats of the intact OCT-1 motif (TGATTTGCATAGCCCTGTGG) or mutated OCT-1 motif (TGGCCTGCATAGCCCTGTGG) upstream of a minimal polyomavirus early promoter linked to a CAT gene via *Hin*dIII- and *Pst*I-cloning sites. In the case of PG-CAATwt or PG-CAATmut, 3 repeats of the intact CAAT motif (TTAACCAATCAC) or mutated CAAT box (TTAACGTATCAC) were cloned into the same reporter plasmids described above.

Cell Culture and Treatment—The human breast carcinoma MCF-7 line, the human lung carcinoma line H1299, and the human colorectal carcinoma line HCT116 were grown in F-12 medium supplemented with 10% fetal bovine serum as described previously (18, 19). For MMS treatment, cells were exposed to medium containing MMS (Aldrich) at 100 μg/ml for 4 h, and then the medium was replaced with fresh medium. For UV radiation, cells in 100-mm dishes were rinsed with PBS and irradiated to a dose of 10 Jm^{-2}. Cells treated with MMS and UV were collected 16 h posttreatment for the CAT assay (20, 54).

Transfection and CAT Assay—4 μg of the *GADD45* promoter reporter constructs and 4 μg of indicated expression vectors were cotransfected into human cells by calcium phosphate precipitation. 40 h later, cells were collected for the CAT assay. In addition, 4 μg of pCMV-GFP plasmid (which expresses green fluorescence protein) was included in each experiment. After transfection, expression of GFP protein was detected by Western blotting assay to determine transfection efficiency. Measurement of CAT activity was carried out as described previously (56). Cells were collected, resuspended in 0.25 M Tris (pH 7.8), and disrupted by three freeze-thaw cycles. Equal amounts of protein were used for each CAT assay. The CAT reaction mixture was incubated at 37 °C overnight, and the CAT activity was determined by measuring the acetylation of ^{14}C-labeled chloramphenicol using thin layer chromatography. Radioactivity was measured directed with Betascope analyzer. The specific CAT activity was calculated by determining the fraction of chloramphenicol that had been acetylated. The relative CAT activity was determined by normalizing the activity of the treated samples to that of the untreated sample. Each value represented the average of at least three separate determinations (54, 56).

Antibodies, Preparation of Nuclear Protein, Immunoprecipitation, and Immunoblotting Analysis—Antibodies against BRCA1, Oct-1, NF-YA, and Jun-D were commercially provided by Santa Cruz Biotechnology (Santa Cruz, CA). For preparation of nuclear protein, exponentially growing HCT116 cells were collected, rinsed with PBS, and resuspended in 200 μl of cold buffer A (10 mM Hepes (pH 7.9); 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride). Following vortexing, the samples were incubated on ice for 10 min, and Nonidet P-40 was added to a final concentration of 0.5%. After centrifugation, insoluble pellets were resuspended in 100 μl of ice-cold buffer C (20 mM Hepes (pH 7.9); 400 mM KCl; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride). The samples were placed on ice and subjected to vortexing for 15 s every 10 min, over a period of 40 min. Finally, the samples were centrifuged at 14,000 × *g* for 10 min, and the supernatant (nuclear extract) was collected for further analysis. For immunoprecipitation and immunoblotting analysis, 300 μg of nuclear protein was immunoprecipitated with anti-BRCA1, Oct-1, NF-YA, or Jun-D antibodies and protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4 °C. The immunoprecipitated protein complexes were washed three times with lysis buffer and loaded onto a SDS-PAGE gel. After electrophoresis, the proteins were transferred to Protran membranes. Membranes were blocked in 5% milk, washed with PBST (PBS with 0.1% Tween), and incubated with anti-Oct-1, NF-YA, and BRCA1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Following washing and incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody at 1:4000 in 5% milk, the membranes were washed, and bound horseradish peroxidase was detected by ECL (Amersham Biosciences) and exposure to x-ray film.

Biotin-Streptavidin Pull-down Assay—Four oligonucleotides containing biotin on the 5′-nucleotide of the sense strand were used in the pull-down assays. The sequences of these oligonucleotides are as follows: 1) wt oligo, 5′-GCAGGCTGATTTGCATAGCCCAATGGCCAAGCTGCATGCAAATGAGGCGGA, which corresponds to positions −107 to −57 of the human *GADD45* promoter; 2) mut oligo1, 5′-GCAGGCTGATTTGCATAGCCtgATGGCCAAGCTGCATGCAAATGAGGCGGA, which corresponds to positions −107 to −57 of the human *GADD45* promoter with the CAAT box mutated; 3) mut oligo2, 5′-GCAGGCTGgccTGCATAGCCCAATGGCCAAGCTGCATGCAggcGAGGCGGA, which corresponds to positions −107 to −57 of the human *GADD45* promoter with two OCT-1 motifs mutated; and 4) mut oligo3, 5′-GCAGGCTGATTTGCATAGCCtgATGGCCAAGCTGCATGCA ggcGAGGCGGA, which corresponds to positions −107 to −57 of the human *GADD45* promoter with two OCT-1 sites and one CAAT box mutated.

These oligonucleotides were annealed to their respective complementary oligonucleotides, and 51-bp double-stranded oligonucleotides were gel-purified and used. Nuclear protein was extracted as described earlier. One microgram of each double-stranded oligonucleotide was incubated with 300 μg of nuclear protein for 20 min at room temperature in binding buffer containing 12% glycerol, 12 mM Hepes (pH 7.9), 4 mM Tris (pH 7.9), 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 10 μg of poly(dI-dC) competitor. Following the incubation, 30 μl of streptavidin-agarose (Sigma) was added to the reaction and incubated at 4 °C for 4 h. Prior to this step, 300 μl of the original streptavidin-agarose bead preparation was preabsorbed with 500 μl of bovine serum albumin, 50 μg of poly(dI-dC), and 50 μg of sheared salmon sperm DNA for 30 min at 25 °C. The streptavidin-agarose beads were washed three times and resuspended in 300 μl of the binding buffer. The protein-DNA-streptavidin-agarose complex was washed three times with binding buffer and loaded onto a SDS gel. Detection of BRCA1, Oct-1, and NF-YA proteins was performed as described above (54).

RESULTS

Mapping of the BRCA1 Regulatory Elements in the GADD45 Promoter—Our group recently demonstrated (41) that BRCA1 induces expression of *GADD45* mRNA and activates the *GADD45* promoter. As shown in Fig. 1*A*, when pHG45-CAT2, a *GADD45* promoter reporter construct that spans −909 to +144

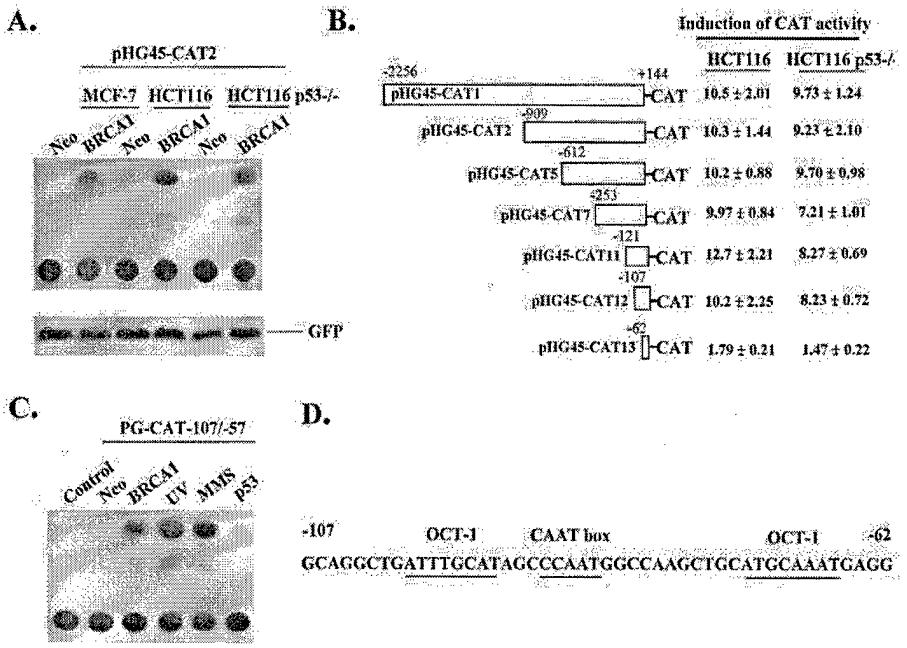

FIG. 1. **Mapping of the BRCA1-regulatory elements in the *GADD45* promoter.** *A*, 4 μg of the *GADD45* promoter CAT reporter constructs pHG45-CAT2 was cotransfected with 4 μg of either pCR3.BRCA1 (BRCA1) or pCMV.neo (Neo) expression vectors into MCF-7, HCT116, or HCT116p53−/− cells using calcium phosphate precipitation. 48 h later, cells were collected, and CAT activity was assayed (see "Experimental Procedures"). To determine transfection efficiency, 4 μg of GFP expression vector was cotransfected with each tested plasmid, and the expression of GFP protein was detected as the internal control of transfection. *B*, 4 μg of the CAT reporter constructs containing the indicated regions of the *GADD45* promoter were cotransfected with pCR3.BRCA1 into HCT116 and HCT116 p53−/− cell lines. CAT assay was carried out as in *A*. *C*, 4 μg of PG-CAT-107/−57 plasmid, where the DNA fragment corresponding to −107 to −57 of the *GADD45* promoter was cloned upstream of a minimal polyomavirus promoter linked to a CAT gene, was cotransfected with either pCR3.BRCA1 (BRCA1), pCMV.neo (Neo), or pC53-SN3 (p53). The CAT assays were performed, and the CAT activities were measured as described under "Experimental Procedures." In some cases, the cells transfected with PG-CAT−107/−57 alone were treated with UV radiation or MMS and followed by CAT assay. All experiments presented in *A* and *C* were repeated at least three times, but only a representative experiment of CAT assay is shown here. *D*, DNA sequence analysis indicates that there are two OCT-1 sites and one CAAT box located at the region of the *GADD45* promoter from −107 to −62.

of the *GADD45* promoter region, was cotransfected with either pCMV.neo (Neo) or pCR3.BRCA1 (BRCA1) into the human breast carcinoma MCF-7 cell line (wt p53), human colorectal carcinoma HCT116 cell line (wt p53), or HCT116 p53−/− cell line (where p53 alleles were deleted via homologue recombination), the *GADD45* promoter reporter was strongly activated in all cell lines regardless of p53 status. To determine transfection efficiency, GFP expression vector was cotransfected with each tested plasmid. The expression of GFP protein detected by immunoblotting analysis indicated that transfection efficiency was similar among different samples with variations less than 20%. To map the BRCA1-responsive elements in the *GADD45* promoter, a series of the *GADD45* CAT reporters that spanned the different regions of the human *GADD45* promoter were constructed. Following cotransfection of these *GADD45* promoter reporter plasmids with the BRCA1 expression vector into human colorectal carcinoma HCT116 and HCT116 p53−/− cells, CAT assays were conducted, and the CAT activities were analyzed. As illustrated in Fig. 1*B*, most of the *GADD45* CAT reporters were strongly activated following expression of BRCA1 protein. With progressive 5′-deletion, pHG45-CAT13 that extended 5′ only to −62 relative to the transcription start site exhibited little induction following expression of BRCA1. These observations indicate that the region between −107 and −62 contains the regulatory elements required for the responsiveness of the *GADD45* promoter to BRCA1 expression.

To confirm if the region from −107 to −62 is responsible for activation of the *GADD45* promoter by BRCA1, we constructed a reporter plasmid designated as PG-CAT−107/−57, where a DNA fragment corresponding to the *GADD45* promoter region between −107 and −57 was cloned upstream of a minimal polyomavirus promoter linked to a CAT reporter gene. This minimal polyomavirus promoter itself is unable to respond to BRCA1 expression or DNA-damaging agents (data not shown). When cotransfected with pCR3.BRCA1 (BRCA1) into HCT116 cells, PG-CAT−107/−57 exhibited induction (Fig. 1*C*). In contrast, both pCMV.neo (Neo) and pC53-SN3 (p53) had no effect on this reporter, indicating that the region between −107 and −57 is capable of conferring the BRCA1 inducibility to a nonrelated promoter reporter. Interestingly, PG-CAT−107/−57 was also shown to be strongly induced by UV radiation and MMS, suggesting that activation of the *GADD45* promoter by BRCA1 and DNA damage might share some common regulatory elements. Inspection of DNA sequence exhibits two OCT-1 motifs and one CAAT box located at this region of the human *GADD45* promoter (Fig. 1*D*).

BRCA1 Activation of the GADD45 Promoter Is Mediated through Both OCT-1 and CAAT Motifs—To determine whether the OCT-1 and CAAT box motifs play roles in activating the *GADD45* promoter following expression of BRCA1, we mutated the OCT-1 or CAAT motifs in *GADD45* promoter CAT reporter constructs (53). It should be noted here that our previous work (54) has demonstrated that there are certain regulatory elements located more upstream of the *GADD45* promoter, such as EGR1/WT1. Therefore, to exclude the influence of such responsive elements, we choose pHG45-CAT11, which only contains the region from −121 to +144 of the *GADD45* promoter. Following cotransfection of these mutants of the *GADD45* promoter reporters into both HCT116 (wt p53) and H1299 cells, where the p53 gene is deleted, induction of CAT activity was determined. As shown in Fig. 2, pHG45-CAT11 exhibited the strongest activation by BRCA1. Single mutation in either OCT-1 or CAAT1 motifs (pHG45-CAT11 m1, pHG45-CAT11 m2, and pHG45-CAT11 m3) had little effect on BRCA1-induced

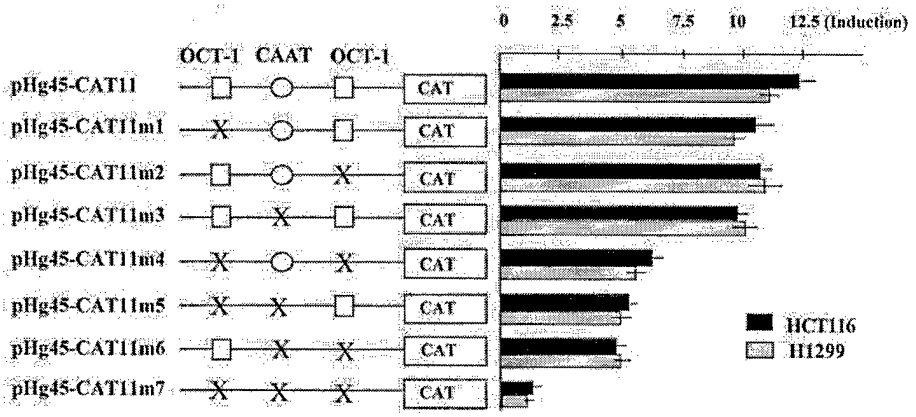

FIG. 2. **Mutations of OCT-1 and CAAT motifs abrogate the activation of the *GADD45* promoter following expression of BRCA1.** 4 μg of the *GADD45* promoter reporter constructs containing the indicated mutations either in OCT-1 sites or in CAAT box were cotransfected with pCR3.BRCA1 into either human colorectal carcinoma HCT116 cells (wt p53) or human lung carcinoma H1299 cells, which contain deleted p53 gene. 40 h later, cells were collected for CAT assay as described under "Experimental Procedures." The values represent the relative induction of the *GADD45* promoter CAT reporters by BRCA1 to that of the Neo-cotransfected controls.

activation of the *GADD45* promoter. However, double mutations in OCT-1 and CAAT sites (pHG45-CAT11 m4, pHG45-CAT11 m5, and pHG45-CAT11 m6) inhibited activation of the *GADD45* promoter by BRCA1, reducing induction of these reporters by 60%. When all three sites were mutated (pHG45-CAT11 m7), the *GADD45* promoter reporter did not exhibit any activation following expression of BRCA1. The responsiveness of the pHG45-CAT11 m7 to BRCA1 expression was observed to be similar to that seen in pHG45-CAT13 (Fig. 1*B*), which only contains the *GADD45* promoter region from −62 to +144. In addition to HCT116 and H1299, we have also examined the activity of the *GADD45* promoter reporters in MCF-7 (wt 53) and HCT116 p53−/− and obtained similar results (data not shown), suggesting that the OCT-1 and CAAT-mediated BRCA1 activation of the *GADD45* promoter does not require p53. These results indicate that both the OCT-1 and CAAT motifs play an important role in BRCA1 activation of the *GADD45* promoter in a p53-independent manner.

We also made mutations in all OCT1 and CAAT motifs in pHG45-CAT2, which covers a longer promoter region between −909 and +144 and determined the BRCA1 activation on this construct. BRCA1 activation of this mutated promoter (pHG45-CAT2ma) was reduced by 70% compared with the pHG45-CAT2 that contains the intact *GADD45* promoter (results not shown). In contrast, BRCA1 activation of the pHG45-CAT11 m7 was completely abolished (Fig. 2). This result is in agreement with our previous finding (54) that there are certain regulatory elements (such as EGR1/WT1) at the upstream region of the *GADD45* promoter. These upstream-responsive elements might also play a role in activation of the *GADD45* promoter by BRCA1, even when mutations were made in OCT1 and CAAT1 motifs.

To determine further the roles of the OCT-1 and CAAT1 motifs in the BRCA1-mediated transcriptional activation, we constructed both OCT-1 and CAAT reporter plasmids, where the multiple repeats of either OCT-1 or CAAT motifs were placed upstream of a polyomavirus minimal promoter that is linked to a chloramphenicol acetyltransferase (CAT) gene. In Fig. 3*A*, PG-OCT-1wt that contains 5 repeats of the intact OCT-1 motifs was transfected with expression vectors for BRCA1, Neo, and Oct-1 into HCT116 cells. PG-OCT-1wt was activated following expression of BRCA1. As an OCT-1 reporter, this construct was also strongly induced by Oct-1 expression. Interestingly, the OCT-1 reporter was responsive to MMS treatment. In contrast, the PG-OCT-1mut that contains 5 repeats of the mutated OCT-1 sites did not exhibit any responsiveness to expression of BRCA1 and Oct-1 protein or to MMS treatment. Similarly, the PG-CAATwt with 3 repeats of the CAAT motifs demonstrated a clear induction following expression of either BRCA1 or NF-YA, which is one of the subunits of NF-Y transcription factor and binds to CAAT box. PG-CAAT

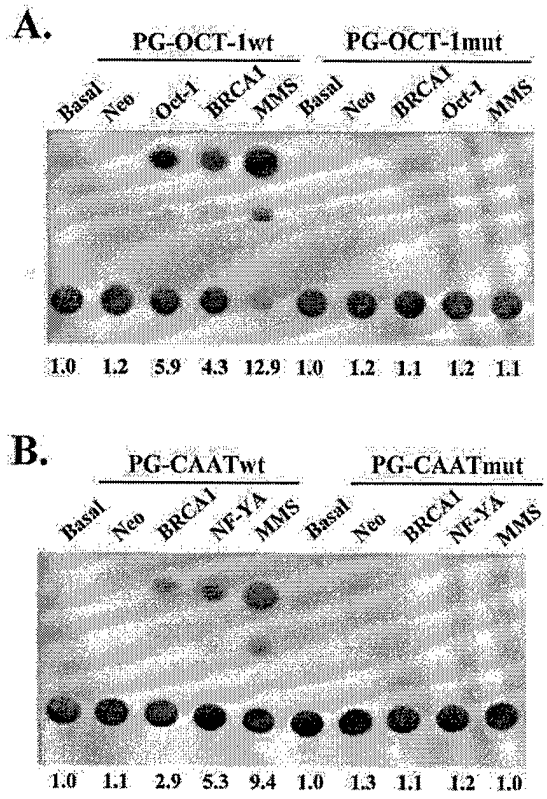

FIG. 3. **Both OCT-1 and CAAT box motifs confer inducibility of BRCA1 to a non-related minimal promoter.** *A*, 4 μg of PG-OCT-1wt and PG-OCT-1mut constructs containing 5 repeats of intact OCT-1 or mutant OCT-1 motifs upstream of the minimal polyomavirus promoter linked to a CAT gene were cotransfected with 4 μg of the indicated expression vectors (pCMV.neo, pCR3.BRCA1, and pCR3.Oct-1) into HCT116 cells. 40 h later, cells were collected for CAT assay as described in *A*. *B*, 4 μg of PG-CAATwt or PG-CAATmut plasmids, which are CAAT reporter constructs (see "Experimental Procedures"), were transfected with 4 μg of the indicated expression vectors (pCMV.neo, pCR3.BRCA1, and pCMV.NF-YA) into HCT116 cells. CAT assay was performed as in *A*.

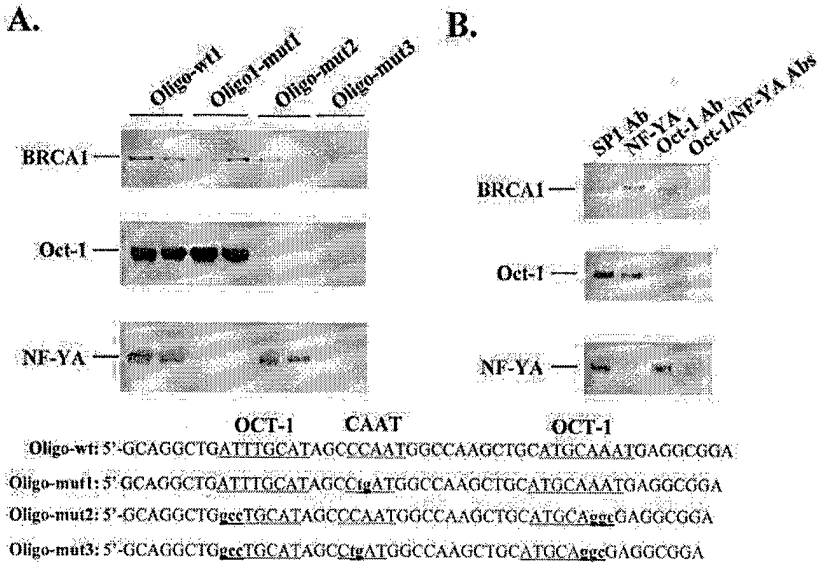

FIG. 4. **Pull-down assay with biotin-labeled oligonucleotides containing the OCT-1 and CAAT1 motifs.** *A*, nuclear extracts were prepared from HCT116 cells as described under "Experimental Procedures" and incubated with biotin-labeled 51-bp oligonucleotides, which contain either intact or mutated OCT-1 and CAAT sequences. Proteins bound to these nucleotides were isolated with streptavidin-agarose beads, and BRCA1, Oct-1, and NF-YA were detected by immunoblotting analysis (see "Experimental Procedures"). *B*, the nuclear extracts were immunodepleted with the antibodies against Jun-D, Oct-1, and NF-YA prior to incubation with the nucleotide containing intact OCT-1 and CAAT motifs (*Oligo-wt*).

also exhibited strong activation by MMS. However, PG-CAAT-mut with mutated CAAT motifs did not respond to expression of BRCA1 and NF-YA or MMS treatment. Collectively, the results presented above further indicate that the BRCA1 activation of the *GADD45* promoter is mediated through the OCT-1 and CAAT motifs.

BRCA1 Physically Interacts with OCT-1 and CAAT Motifs Via Its Physical Association with Both Oct-1 and NF-YA Proteins—Because the OCT-1 and CAAT motifs mediate the transcriptional activation of the *GADD45* promoter by BRCA1, effort was made to determine whether BRCA1 directly binds to the *GADD45* promoter region containing both OCT-1 and CAAT sites. An approach called "biotin-streptavidin pull-down assay" was employed to identify the proteins bound to the BRCA1-responsive region of the *GADD45* promoter. The biotin-labeled 51-bp double-stranded oligonucleotides corresponding to −107 to −57 of the *GADD45* promoter were incubated with nuclear extracts from HCT116 cells and pulled down by streptavidin (see "Experimental Procedures"). The protein complexes bound to the oligonucleotides were loaded onto SDS-PAGE gel and analyzed by immunoblotting assay with antibodies against BRCA1, Oct-1, and NF-YA. In Fig. 4*A*, the Oligo-wt that contains the intact OCT-1 and CAAT motifs was able to pull down the Oct-1, NF-YA, and BRCA1 proteins, indicating that all three proteins physically associate with this BRCA1-regulatory region. In Oligo-mut1, where the CAAT box was mutated, both the Oct-1 and BRCA1 proteins but not NF-YA were detected in the precipitated complexes. In the case of Oligo-mut2, where two OCT-1 sites were disrupted, BRCA1 and NF-YA proteins were present but not Oct-1. However, when all OCT-1 and CAAT motifs were mutated in the Oligo-mut3, no BRCA1, Oct-1, or NY-FA proteins were detected. These results strongly suggest the following two interpretations: (*a*) BRCA1 physically associates with the region of the *GADD45* promoter between −107 and −57 through its interaction with both OCT-1 and CAAT motifs; and (*b*) BRCA1 interacts with OCT-1 or CAAT motifs independently because single mutation of either motif did not disrupt BRCA1 interaction with the BRCA1-responsive region of the *GADD45* promoter.

However, because BRCA1 is not a sequence-specific binding transcription factor, it is most likely that the association of BRCA1 protein with the *GADD45* promoter is through its interaction with the Oct-1 and NF-Y factors, which directly bind to the *GADD45* promoter via their motifs. To address this

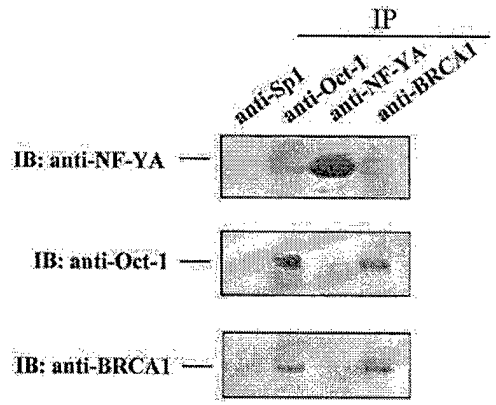

FIG. 5. **Physical association of BRCA with Oct-1 and NF-YA.** Nuclear protein from HCT116 cells was prepared (see "Experimental Procedures") and immunoprecipitated with anti-Jun-D, anti-Oct-1, anti-NF-YA, and anti-BRCA1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The immunocomplexes were analyzed by SDS-PAGE and immunoblotted with antibodies against NF-YA, Oct-1, and BRCA1, respectively. The visualized bands are shown; their estimated masses were 42–46 kDa for NF-YA, 97 kDa for Oct-1, and 220 kDa for BRCA1. *IP*, immunoprecipitation; *IB*, immunoblotting analysis.

issue, the Oligo-wt was incubated with the nuclear extracts, which were immunodepleted with anti-Oct-1 or -NF-YA antibodies prior to the pull-down assay. As shown in Fig. 4*B*, depletion with single antibody to either Oct-1 or NF-YA proteins did not affect binding of BRCA1 to the *GADD45* promoter region. However, immunodepletion of both the Oct-1 and NF-YA proteins completely abolished the association of BRCA1 with the *GADD45* promoter, indicating that association of BRCA1 with the *GADD45* promoter is through its interaction with the Oct-1 and NF-YA proteins, which directly bind to the *GADD45* promoter.

Next, we further determined the physical interactions of BRCA1 with Oct-1 and NF-YA proteins. Nuclear extracts isolated from HCT116 cells were incubated with anti-Jun-D, anti-Oct-1, anti-NF-YA, or anti-BRCA1 antibodies and immunoprecipitated with protein A/G-agarose beads. The immunocomplexes were then analyzed by Western blotting assay, and the results are shown in Fig. 5. NF-YA protein was present in the immunocomplexes precipitated by the antibodies against Oct-1, NF-YA, and BRCA1, suggesting physical interactions of NF-YA with Oct-1 and BRCA1. Oct-1 protein was detected in

the immunocomplexes with both anti-Oct-1 and anti-BRCA1 antibodies. Similarly, BRCA1 protein was detected in the anti-Oct-1 and anti-BRCA1 immunocomplexes. In contrast, no NF-YA, Oct-1, or BRCA1 proteins was present in the anti-Jun-D-immunoprecipitated complex. However, it is somewhat surprising that we did not detect Oct-1 and BRCA1 proteins in the anti-NF-YA-immunocomplex. One likely interpretation is that the interacting domains of Oct-1 and BRCA1 in NF-YA protein might share the region with the epitopes to the antibody against NF-YA, which possibly lead to dissociation of the NF-YA-BRCA1 and NF-YA-Oct-1 protein complexes. Taken together, these results indicate an association of BRCA1 with Oct-1 and NF-YA and an interaction between Oct-1 and NF-YA as well.

DISCUSSION

Studies presented in this paper and our earlier report (41) have demonstrated that BRCA1 activates the *GADD45* promoter. By using 5′-deletion analysis, the BRCA1-regulatory elements have been mapped at the *GADD45* promoter region between −107 and −62, where there are two OCT-1 motifs and one CAAT motif. Disruption of the OCT-1 and CAAT motifs abrogates the activation of the *GADD45* promoter by BRCA1 expression, indicating that both OCT-1 and CAAT sites are required for the BRCA1 activation of the *GADD45* promoter. This finding is further supported by the observation that the OCT-1 and CAAT motifs are able to confer BRCA1 inducibility to a non-related minimal polyomavirus promoter, when multiple repeats of these motifs are cloned upstream of the minimal promoter linked to a CAT gene. In the biotin-streptavidin pull-down assay, BRCA1 protein exhibits an association with the oligonucleotides corresponding to the *GADD45* promoter region from −107 to −57. Mutations of all OCT-1 and CAAT sites in such oligonucleotides disrupt association of BRCA1 with the *GADD45* promoter. Importantly, BRCA1 protein is demonstrated to interact physically with both Oct-1 and NF-YA proteins, and depletion of Oct-1 and NF-YA proteins results in abrogation of association of BRCA1 with the *GADD45* promoter. We conclude that BRCA1 transactivation of the *GADD45* promoter is mediated through BRCA1 interaction with Oct-1 and NF-YA proteins.

BRCA1 has been implicated in DNA damage-induced cellular response, including apoptosis, cell cycle arrest, and DNA repair (7–13). Inactivation of *BRCA1* correlates with genomic instability (14), indicating that one of the major roles for *BRCA1* is to maintain genomic fidelity. In addition to direct interactions of BRCA1 with proteins involved in cell cycle control and DNA repair, BRCA1-mediated transcriptional regulation may also greatly contribute to its role in cellular response to DNA damage. For example, both p21and *GADD45*, which are important players in the control of cell cycle checkpoints (29, 57), are regulated by BRCA1 (10, 41). It has been well accepted that the roles of BRCA1 as a tumor suppressor might be at least in part mediated through its transcriptional properties, given the evidence that tumor-derived mutations within the C terminus of BRCA1 are defective in transcriptional activation (10, 32). In agreement with this point, the tumor-derived BRCA1 mutants (p1749R and Y1853insA) that lack transcriptional activity are unable to activate the *GADD45* promoter (41). However, the regulation of *GADD45* by BRCA1 appears to be complex and might involve differential mechanism(s). This complex regulation may be due to the following points. (*a*) BRCA1 activation of *GADD45* has been shown to involve the BRCA1-responsive elements located at both the intronic or promoter regions of *GADD45* (30, 41, 58). (*b*) Most likely, BRCA1 regulates *GADD45* through its interaction with other transcription factors that directly bind to the *GADD45* promoter or intronic regions instead of direct binding of BRCA1 to the regulatory regions. (*c*) BRCA1 protein might be subject to phosphorylation in the process of DNA damage-induced transcriptional activation (5, 6). (*d*) BRCA1-mediated transactivation might recruit transcriptional coactivators, such as p300/CBP (38). Therefore, future work will further characterize the biochemical consequences of the interaction between BRCA1 and Oct-1 and NF-YA to determine whether Oct-1 and NF-YA are subject to protein stabilization, phosphorylation, or acetylation.

The *GADD45* promoter is strongly activated following genotoxic stress, including UV radiation, MMS, and medium starvation (54). Most recently, we have demonstrated that the p53-independent UV induction of the *GADD45* promoter is also regulated through both OCT-1 and CAAT motifs located at the same region between −107 and −62 of the *GADD45* promoter. Mutations of all OCT-1 and CAAT motifs abolish the induction of the *GADD45* promoter by UV radiation and MMS. In addition, protein levels of the Oct-1 and NF-YA transcription factors are elevated following DNA damage (53). Moreover, mitogen-activated protein kinases (c-Jun N-terminal kinase and extracellular signal-regulated kinase) also activate the *GADD45* promoter through the OCT-1 and CAAT motifs. In the current study, we demonstrate that the OCT-1 and CAAT motifs mediate the BRCA1 activation of the *GADD45* promoter. Therefore, it can be speculated that the OCT-1 and CAAT motifs are critical in the regulation of the p53-independent induction of *GADD45* in response to growth arrest signals (such as BRCA1 expression) and a variety of DNA-damaging agents. It is worth noting that in the OCT-1 and CAAT motifs appear to function in an additive but independent manner because single mutation of either OCT-1 sites or the CAAT box only reduced induction of the *GADD45* promoter by BRCA1, whereas mutations of all OCT-1 and CAAT motifs completely disrupted the BRCA1 activation of the *GADD45* promoter (Fig. 2).

The finding that BRCA1 regulates the *GADD45* through its interaction with transcription factors Oct-1 and NF-YA is of importance, given evidence that both the OCT-1 and CAAT motifs are widely present in the many gene promoters. Oct-1 and NF-YA are ubiquitous transcription factors involved in the development, cell cycle regulation, and cellular senescence (50, 51, 59, 60). Recently, we have found that OCT-1 and NF-YA proteins are induced after exposure of cells to multiple DNA-damaging agents and therapeutic agents in a p53-independent manner (52, 53). These observations indicate that both Oct-1 and NF-YA proteins are able to participate in cellular responses to genotoxic stress. In addition, our current study has shown a physical interaction of NF-YA with Oct-1 protein, suggesting that induction of *GADD45* by BRCA1 might involve a functional interaction between these two proteins. In fact, Oct-1 and NF-YA proteins have been reported previously to synergistically regulate histone H2B gene transcription during *Xenopus* early development (61). In summary, the study presented here has demonstrated the biochemical mechanism by which BRCA1 regulates the *GADD45* promoter and indicated that *GADD45* is a BRCA1 downstream effector. Furthermore, identification of the OCT-1 and CAAT1 as BRCA1-responsive elements has broadened the biological roles for *BRCA1* in transcriptional regulation.

REFERENCES

1. Easton, D. F., Ford, D., and Bishop, D. T. (1995) *Am. J. Hum. Genet.* **56**, 265–271
2. Ford, D., and Easton, D. F. (1995) *Br. J. Cancer* **72**, 805–812
3. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., *et al.* (1994) *Science* **266**, 66–71
4. Marquis, S. T., Rajan, J. V., Wynshaw-Boris, A., Xu, J., Yin, G. Y., Abel, K. J.,

Weber, B. L., and Chodosh, L. A. (1995) *Nat. Genet.* **11,** 17–26
5. Cortez, D., Wang, Y., Qin, J., and Elledge, S. J. (1999) *Science* **286,** 1162–1166
6. Lee, J. S., Collins, K. M., Brown, A. L., Lee, C. H., and Chung, J. H. (2000) *Nature* **404,** 201–204
7. Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., and Livingston, D. M. (1997) *Cell* **88,** 265–275
8. Gowen, L. C., Avrutskaya, A. V., Latour, A. M., Koller, B. H., and Leadon, S. A. (1998) *Science* **281,** 1009–1012
9. Larson, J. S., Tonkinson, J. L., and Lai, M. T. (1997) *Cancer Res.* **57,** 3351–3355
10. Somasundaram, K., Zhang, H., Zeng, Y. X., Houvras, Y., Peng, Y., Wu, G. S., Licht, J. D., Weber, B. L., and El-Deiry, W. S. (1997) *Nature* **389,** 187–190
11. Shao, N., Chai, Y. L., Shyam, E., Reddy, P., and Rao, V. N. (1996) *Oncogene* **13,** 1–7
12. Fan, S., Wang, J. A., Yuan, R. Q., Ma, Y. X., Meng, Q., Erdos, M. R., Brody, L. C., Goldberg, I. D., and Rosen, E. M. (1998) *Oncogene* **16,** 3069–3082
13. Thangaraju, M., Kaufmann, S. H., and Couch, F. J. (2000) *J. Biol. Chem.* **275,** 33487–33496
14. Shen, S. X., Weaver, Z., Xu, X., Li, C., Weinstein, M., Chen, L., Guan, X. Y., Ried, T., and Deng, C. X. (1998) *Oncogene* **17,** 3115–3124
15. Fornace, A. J., Jr., Alamo, I., Jr., and Hollander, M. C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8800–8804
16. Fornace, A. J., Jr., Papathanasiou, M. A., Tarone, R. E., Wong, M., Mitchell, J., and Hamer, D. H. (1990) *Prog. Clin. Biol. Res.* **340A,** 315–325
17. Papathanasiou, M. A., Kerr, N. C., Robbins, J. H., McBride, O. W., Alamo, I., Jr., Barrett, S. F., Hickson, I. D., and Fornace, A. J., Jr. (1991) *Mol. Cell. Biol.* **11,** 1009–1016
18. Kastan, M. B., Zhan, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. (1992) *Cell* **71,** 587–597
19. Zhan, Q., Bae, I., Kastan, M. B., and Fornace, A. J., Jr. (1994) *Cancer Res.* **54,** 2755–2760
20. Zhan, Q., Fan, S., Smith, M. L., Bae, I., Yu, K., Alamo, I., Jr., O'Connor, P. M., and Fornace, A. J., Jr. (1996) *DNA Cell Biol.* **15,** 805–815
21. Zhan, Q., Lord, K. A., Alamo, I., Jr., Hollander, M. C., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D. A., and Fornace, A. J., Jr. (1994) *Mol. Cell. Biol.* **14,** 2361–2371
22. Jin, S., Antinore, M. J., Lung, F. D., Dong, X., Zhao, H., Fan, F., Colchagie, A. B., Blanck, P., Roller, P. P., Fornace, A. J., Jr., and Zhan, Q. (2000) *J. Biol. Chem.* **275,** 16602–16608
23. Smith, M. L., Chen, I. T., Zhan, Q., Bae, I., Chen, C. Y., Gilmer, T. M., Kastan, M. B., O'Connor, P. M., and Fornace, A. J., Jr. (1994) *Science* **266,** 1376–1380
24. Kearsey, J. M., Coates, P. J., Prescott, A. R., Warbrick, E., and Hall, P. A. (1995) *Oncogene* **11,** 1675–1683
25. Zhao, H., Jin, S., Antinore, M. J., Lung, F. D., Fan, F., Blanck, P., Roller, P., Fornace, A. J., Jr., and Zhan, Q. (2000) *Exp. Cell Res.* **258,** 92–100
26. Zhan, Q., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., and Fornace, A. J., Jr. (1999) *Oncogene* **18,** 2892–2900
27. Carrier, F., Georgel, P. T., Pourquier, P., Blake, M., Kontny, H. U., Antinore, M. J., Gariboldi, M., Myers, T. G., Weinstein, J. N., Pommier, Y., and Fornace, A. J., Jr. (1999) *Mol. Cell. Biol.* **19,** 1673–1685
28. Takekawa, M., and Saito, H. (1998) *Cell* **95,** 521–530
29. Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O'Connor, P. M., Fornace, A. J., Jr., and Harris, C. C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3706–3711
30. Harkin, D. P., Bean, J. M., Miklos, D., Song, Y. H., Truong, V. B., Englert, C., Christians, F. C., Ellisen, L. W., Maheswaran, S., Oliner, J. D., and Haber, D. A. (1999) *Cell* **97,** 575–586
31. Hollander, M. C., Sheikh, M. S., Bulavin, D. V., Lundgren, K., Augeri-Henmueller, L., Shehee, R., Molinaro, T. A., Kim, K. E., Tolosa, E., Ashwell, J. D., Rosenberg, M. P., Zhan, Q., Fernandez-Salguero, P. M., Morgan, W. F., Deng, C. X., and Fornace, A. J., Jr. (1999) *Nat. Genet.* **23,** 176–184
32. Chapman, M. S., and Verma, I. M. (1996) *Nature* **382,** 678–679
33. Ouchi, T., Monteiro, A. N., August, A., Aaronson, S. A., and Hanafusa, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2302–2306
34. Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Bi, D., Weber, B. L., and El-Deiry, W. S. (1998) *Oncogene* **16,** 1713–1721
35. Wang, Q., Zhang, H., Kajino, K., and Greene, M. I. (1998) *Oncogene* **17,** 1939–1948
36. Ouchi, T., Lee, S. W., Ouchi, M., Aaronson, S. A., and Horvath, C. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 5208–5213
37. Fan, S., Wang, J., Yuan, R., Ma, Y., Meng, Q., Erdos, M. R., Pestell, R. G., Yuan, F., Auborn, K. J., Goldberg, I. D., and Rosen, E. M. (1999) *Science* **284,** 1354–1356
38. Pao, G. M., Janknecht, R., Ruffner, H., Hunter, T., and Verma, I. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1020–1025
39. Chen, G. C., Guan, L. S., Yu, J. H., Li, G. C., Choi Kim, H. R., and Wang, Z. Y. (2001) *Biochem. Biophys. Res. Commun.* **284,** 507–514
40. MacLachlan, T. K., Somasundaram, K., Sgagias, M., Shifman, Y., Muschel, R. J., Cowan, K. H., and El-Deiry, W. S. (2000) *J. Biol. Chem.* **275,** 2777–2785
41. Jin, S., Zhao, H., Fan, F., Blanck, P., Fan, W., Colchagie, A. B., Fornace, A. J., Jr., and Zhan, Q. (2000) *Oncogene* **19,** 4050–4057
42. Zheng, L., Pan, H., Li, S., Flesken-Nikitin, A., Chen, P. L., Boyer, T. G., and Lee, W. H. (2000) *Mol. Cell* **6,** 757–768
43. Sturm, R. A., Das, G., and Herr, W. (1988) *Genes Dev.* **2,** 1582–1599
44. Fletcher, C., Heintz, N., and Roeder, R. G. (1987) *Cell* **51,** 773–781
45. LaBella, F., Sive, H. L., Roeder, R. G., and Heintz, N. (1988) *Genes Dev.* **2,** 32–39
46. Murphy, S., Pierani, A., Scheidereit, C., Melli, M., and Roeder, R. G. (1989) *Cell* **59,** 1071–1080
47. Bergman, Y., Rice, D., Grosschedl, R., and Baltimore, D. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 7041–7045
48. Eraly, S. A., Nelson, S. B., Huang, K. M., and Mellon, P. L. (1998) *Mol. Endocrinol.* **12,** 469–481
49. Fadel, B. M., Boutet, S. C., and Quertermous, T. (1999) *J. Biol. Chem.* **274,** 20376–20383
50. Mantovani, R. (1999) *Gene (Amst.)* **239,** 15–27
51. Matuoka, K., and Yu Chen, K. (1999) *Exp. Cell Res.* **253,** 365–371
52. Zhao, H., Jin, S., Fan, F., Fan, W., Tong, T., and Zhan, Q. (2000) *Cancer Res.* **60,** 6276–6280
53. Jin, S., Fan, F., Fan, W., Zhao, H., Tong, T., Blanck, P., Alomo, I., Rajasekaran, B., and Zhan, Q. (2001) *Oncogene* **20,** 2683–2690
54. Zhan, Q., Chen, I. T., Antinore, M. J., and Fornace, A. J., Jr. (1998) *Mol. Cell. Biol.* **18,** 2768–2778
55. Kern, S. E., Pietenpol, J. A., Thiagalingam, S., Seymour, A., Kinzler, K. W., and Vogelstein, B. (1992) *Science* **256,** 827–830
56. Zhan, Q., Carrier, F., and Fornace, A. J., Jr. (1993) *Mol. Cell. Biol.* **13,** 4242–4250
57. Waldman, T., Kinzler, K. W., and Vogelstein, B. (1995) *Cancer Res.* **55,** 5187–5190
58. Li, S., Chen, P. L., Subramanian, T., Chinnadurai, G., Tomlinson, G., Osborne, C. K., Sharp, Z. D., and Lee, W. H. (1999) *J. Biol. Chem.* **274,** 11334–11338
59. Sturm, R. A., Dalton, S., and Wells, J. R. (1988) *Nucleic Acids Res.* **16,** 8571–8586
60. Inamoto, S., Segil, N., Pan, Z. Q., Kimura, M., and Roeder, R. G. (1997) *J. Biol. Chem.* **272,** 29852–29858
61. Hinkley, C., and Perry, M. (1992) *Mol. Cell. Biol.* **12,** 4400–4411

Appendix 5

Abstract for the 93rd Annual Meeting of American Association of Cancer Research

BRCA1 regulates *GADD45* through its interactions with the OCT-1 and CAAT motifs. Wenhong Fan, Shunqian Jin, Tong Tong, Hongcheng Zhao, Feiyue Fan, Patricia Blanck, Isaac Alamo, Baskaran Rajasekaran and Qimin Zhan. Department of Radiation Oncology, Cancer Institute and Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

The implication of BRCA1 in gene regulation greatly accounts for its role as a tumor suppressor. The BRCA1-targeted genes are thought to mediate BRCA1's biological function. Previous studies by others and ours demonstrate that BRCA1 induces *GADD45*, a p53-regulated and stress-inducible gene that plays an important role in cellular response to DNA damage. However, the molecular mechanism(s) by which BRCA1 regulates *GADD45* remains to be elucidated. In this report, we have shown that BRCA1 strongly activates the *GADD45* promoter in a p53-independent manner and the BRCA1 activation of the *GADD45* promoter is mediated through the OCT-1 and CAAT motifs located at the *GADD45* promoter region between –107 and –57. Site-directed mutations of both OCT-1 and CAAT motifs abrogate induction of the *GADD45* promoter by BRCA1. When several repeats of OCT-1 or CAAT are cloned upstream of a minimal polyomavirus promoter linked to a chloramphenicol acetyltransferase (CAT) reporter gene, both OCT-1 and CAAT motifs confer BRCA1 inducibility on the non-related minimal promoter. Physical associations of BRCA1 protein with transcription factors Oct-1 and NF-YA, which directly bind to the OCT-1 and CAAT motifs, are established by biotin-streptavidin pull-down and coimmunoprecipitation assays. Such protein interactions are required for interaction of BRCA1 with the *GADD45* promoter since either immunodepletion of Oct-1 and NF-YA proteins or mutations in the OCT1and CAAT motifs disrupt BRCA1 binding to the *GADD45* promoter. These findings indicate that BRCA1 can upregulate its targeted genes through protein-protein interactions and provide a novel mechanism by which BRCA1 participates in transcriptional regulation.

Appendix 6

Abstract for the 94th Annual Meeting of American Association of Cancer Research

GADD45-induce apoptosis and cell growth suppression

Tong Tong, Shunqian Jin, Wenhong Fan Hongcheng Zhao, Feiyue Fan, Patricia Blanck, Isaac Alamo and Qimin Zhan. Department of Radiation Oncology and Department of Molecular Genetic and biochemistry, Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213.

A number of observations have demonstrated that GADD45, a p53-regulated stress-inducible gene, plays an important role in cellular response to DNA damage. Overexpression of Gadd45 has shown to suppress cell growth in multiple cell line, probably through its role in the control cell cycle arrest and apoptosis. However, the understanding of how GADD45 inhibits cell growth remains to be further elucidated. In the present study, we generated GADD45-inducible cells through tetracycline system (tet-off) in both Hela (inactivated p53) and HCT116 lines (wt p53). Following withdrawal of tetracycline, Gadd45 protein is highly induced in these cell lines. In the colony formation assay, inducible expression of Gadd45 suppresses approximately 90% cell growth in both Hela and HCT116 cells, regardless of p53 status. Interestingly, GADD45-induced G2/M arrest is only observed in HCT116, which contains wt p53 but not seen Hela cells that have inactivated p53. Apparently, the GADD45-associated growth inhibition in Hela cells, where cellular p53 function is disrupted, might utilize differential pathways from cell cycle arrest. Therefore, it is speculated that the GADD45-induced growth suppression in cells harboring abnormal p53 is likely associated with apoptosis. Using tunnel assay and DAP1 staining approach, induced expression of GADD45 is able to result in apoptosis in Hela cells. In agreement with those results, cells expressing high levels of Gadd45 protein exhibit PARP cleavage. Induction of GADD45 leads to moderately activation of JNK kinase but not p38 kinase. However, JNK kinase inhibitor has little effect on GADD45-induced apoptosis and growth suppression, suggesting that GADD45-induced apoptosis and growth suppression in Hela cells might not be mainly through JNK pathway. Furthermore, inducible expression of Gadd45 reveals caspase-9 cleavage but not capase-8 cleavage, indicating that GADD45-induced apoptosis involves the mitochondria-related pathway but not the receptor-mediated pathway. Taken together, GADD45-induced apoptosis greatly contribute to the GADD45-associated growth suppression, particularly in cells with negative p53 status.

Appendix 7

Abstract for the Era of Hope meeting in Florida, 2002

BRCA1 regulates *GADD45* through its interactions with the OCT-1 and CAAT motifs. Wenhong Fan, Shunqian Jin, Tong Tong, Hongcheng Zhao, Feiyue Fan, Patricia Blanck, Isaac Alamo, Baskaran Rajasekaran and Qimin Zhan. Department of Radiation Oncology, Cancer Institute and Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

The implication of BRCA1 in gene regulation greatly accounts for its role as a tumor suppressor. The BRCA1-targeted genes are thought to mediate BRCA1's biological function. Previous studies by others and ours demonstrate that BRCA1 induces *GADD45*, a p53-regulated and stress-inducible gene that plays an important role in cellular response to DNA damage. However, the molecular mechanism(s) by which BRCA1 regulates *GADD45* remains to be elucidated. In this report, we have shown that BRCA1 strongly activates the *GADD45* promoter in a p53-independent manner and the BRCA1 activation of the *GADD45* promoter is mediated through the OCT-1 and CAAT motifs located at the *GADD45* promoter region between –107 and –57. Site-directed mutations of both OCT-1 and CAAT motifs abrogate induction of the *GADD45* promoter by BRCA1. When several repeats of OCT-1 or CAAT are cloned upstream of a minimal polyomavirus promoter linked to a chloramphenicol acetyltransferase (CAT) reporter gene, both OCT-1 and CAAT motifs confer BRCA1 inducibility on the non-related minimal promoter. Physical associations of BRCA1 protein with transcription factors Oct-1 and NF-YA, which directly bind to the OCT-1 and CAAT motifs, are established by biotin-streptavidin pull-down and coimmunoprecipitation assays. Such protein interactions are required for interaction of BRCA1 with the *GADD45* promoter since either immunodepletion of Oct-1 and NF-YA proteins or mutations in the OCT1and CAAT motifs disrupt BRCA1 binding to the *GADD45* promoter. These findings indicate that BRCA1 can upregulate its targeted genes through protein-protein interactions and provide a novel mechanism by which BRCA1 participates in transcriptional regulation.